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TOPICAL APPLICATIONS OF VITAMIN A AND OF CAROTENE

Part I—Absorption of Vitamin A from Halibut-Liver Oil

By AGNES C. HELMER and REV. CORNELIUS H. JANSEN

The application of vitamin A to the skin, especially in the form of cod-liver oil, has often been recorded but no direct proof exists of the absorption of the vitamin through this channel. One of the earliest references to this phase of vitamin activity is found in a report by Darbey in 1789. While no reference is made to the absorption of vitamin A, Darbey states that at the Manchester Infirmary cod-liver oil had been used by physicians to cure rickets and that it was apparently used, mainly as an external remedy, for many years.[1]

In 1927 H. Beck stated that up to his time vitamins were practically always taken orally, though he cites Petenyi as having applied vitamin A rectally in certain forms of intestinal disorders. He also mentioned that Klotz reported rectal applications to be more practical than oral in cases of diabetic glycolysis, while Wilhelm Richter found the local applications of vitamin A in skin diseases very beneficial. Dr. Beck himself used a preparation named Vitrisol, prepared from spinach, with very high vitamin A content, which, in addition, was subjected to a prolonged exposure to ultra-violet light. This preparation he used for acne vulgaris and rosacea, in conjunction with a form of electrotherapy in which the infected area was covered with Vitrisol and one of the lead plate electrodes then pressed over the salve. The same preparation he applied

[1]McCollum and Simmonds, The Newer Knowledge of Nutrition (1929): p. 283, The Macmillan Co., New York.

for eczema with excellent results and likewise for ulcera cruris, where he reported success in curing 60 per cent of the cases.[2]

W. Löhr cites the beneficial effects of cod-liver oil in the treatment of wounds. He states that the ordinary bacteria encountered in infected wounds, streptococci, staphylococci, and colon bacilli perish when introduced into cod-liver oil. He used the oil in combination with indifferent substances to give it the consistency of a paste. The oil in the salve permeated the tissues and caused a rapid liquefaction of the necrotic tissue, followed by a powerful stimulation of growth which affected all the tissues, including the epithelium. The author is of the opinion that the effects enumerated are due to the vitamin A and D content of cod-liver oil.[3]

A little later Horn and Sandor used a preparation of oil which contained 2000 International Units of vitamin A per cubic centimeter. They found that this oil, or salve, containing vitamin A did not irritate a fresh wound, had a favorable reaction upon the healing process, checked secondary infection and notably hastened the formation of granulated tissue and the epithelization of the wound.[4]

From these observations the stimulating effect of vitamin A on surface tissue can be deduced. The lack of vitamin A shows itself principally in disorders of the epithelial layers. The occurrence of dermatoses involving dryness of the skin, itching, folliculitis and acneform eruptions are some of the acute manifestations of vitamin A deficiencies. They involve a condition of dyskeratosis, the principal involvement being the cutaneous epithelium. It is usually accompanied by night blindness and other forms of xerophthalmia.[5]

---

[2]Beck, H., *Münch. med. Wochschr.*, **74** (1927): 1129.

[3]Löhr, W., *Deut. med. Wochschr.*, **60** (1934): 561.

[4]Horn, Z., and Sandor, St., *Deut. med. Wochschr.*, **60** (1934): 1018.

[5]*J. Am. Med. Assoc.*, **102** (1934): 770.

Since the lack of vitamin A manifests itself so frequently in epithelial disorders, a direct application of the vitamin to the skin might perhaps be more effective than its usual introduction by way of mouth. The purpose of these investigations was to determine the absorbability of vitamin A through the skin by topical application.

EXPERIMENTAL

Rats 4 weeks old, weighing about 45 grams each, were used in the experiments. They were fed the following vitamin A-free diet:*

Casein (deactivated)	18 per cent
Salt mixture	4 per cent
Yeast, dried	8 per cent
Starch (wheat)	65 per cent
Vegetable oil	5 per cent
Vitamin D (irradiated ergosterol)	A sufficient amount

The salt mixture was composed as follows:

Sodium chloride	17.3 g.
Anhydrous magnesium sulphate	26.6 g.
Sodium phosphate	34.7 g.
Potassium phosphate	95.4 g.
Calcium acid phosphate	54.0 g.
Ferric citrate	11.8 g.
Calcium lactate	130.0 g.

The animals were kept in screen-bottomed metal cages. During the first 3 weeks the rats were kept in groups of 10 or 12, in large cages, with feed and water present at all times. Each animal was weighed twice a week until the end of the depletion period, at which time they were placed in individual cages and weighed daily.

*U. S. Pharmacopoeia, Eleventh Decennial Revision, 1936.

A rat was considered vitamin A-deficient when its weight was the same as, or less than, the weight 7 days previous.*

No standard tests have been recommended by the United States Pharmacopoeia for topical application of vitamins. Since there was no prescribed technic, the following procedure was adopted: The hair was clipped from an area of approximately 12 sq. cm. on the back of each rat, close to the neck. This area of skin was chosen to make sure that the rats would be unable to lick any oil remaining on the skin and thus perhaps receive their supply of the vitamin by mouth.** Then .05 cc. of halibut-liver oil, containing approximately 2530 U. S. P. units of vitamin A, was applied to this limited area of skin and rubbed in with the finger until all the oil was absorbed.

The rats were divided into 2 groups and each rat was numbered. For 25 days all were fed the vitamin A-deficient diet and during this period were weighed twice each week. From the fifteenth day on, all the rats showed either a loss in weight or the absence of any gain, indicating vitamin A deficiency. At the end of the depletion period the 10 rats in Group I were rubbed daily with the halibut-liver oil for a period of 8 days. Each animal was kept in an individual cage and its weight taken daily.

About one-half showed a gain in weight 2 days after the treatment began, and all showed definite gain at the end of 4 days (Figs. 1-10), while during this same interval the controls (Group II) showed no gain (Figs. 11-15). At the end of 3 weeks, the gain in weight for all the rats in Group I ranged

*U. S. Pharmacopoeia, loc. cit.

**In previous experiments protective means such as tape and jackets were employed to insure against the ingestion of the vitamin. As a result of observations made in these earlier experiments it was found that with our improved technic such protective means were unnecessary and frequently interfered with the health of the animal.

from 50 to 120 per cent, the average for the group being 75 per cent. The controls for the most part, however, continued to lose weight and by the end of the experiment 75 per cent showed signs of xerophthalmia, ranging from mild to severe. At the end of 6 weeks all control animals were dead. Of the 10 rats to which halibut-liver oil had been applied, none died. One rat showed signs of xerophthalmia 2 days before the first oil treatment. On the sixth day of the treatment the disorder had become severe, but thereafter rapidly diminished, and on the tenth day the animal was wholly cured.

Growth Curves of Rats to which Halibut-Liver Oil was Topically Applied

FIG. 1

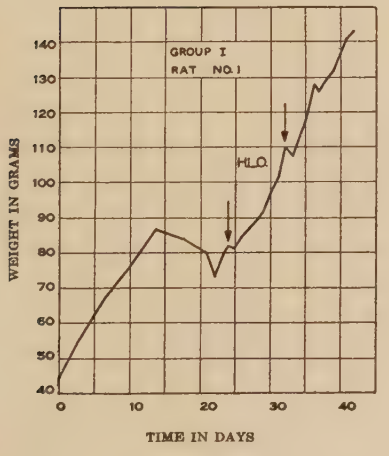
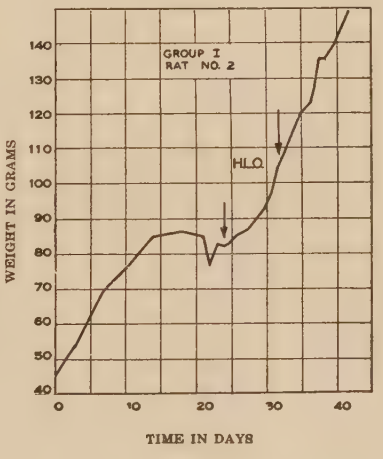


FIG. 2



Space between arrows indicates the interval during which the vitamin was applied.

Growth Curves of Rats to which Halibut-Liver Oil was Topically Applied

FIG. 3

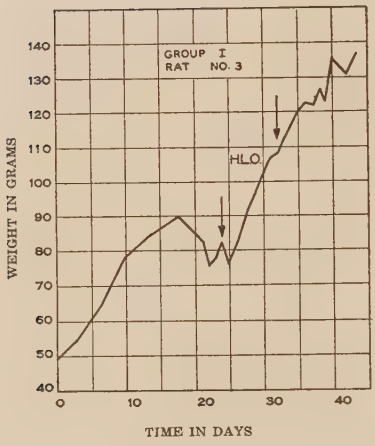


FIG. 4

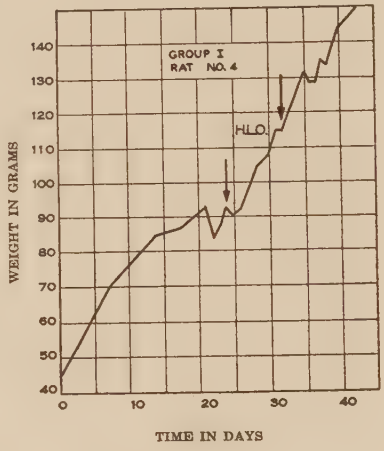


FIG. 5

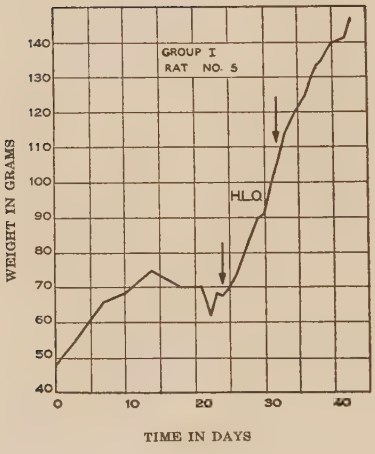
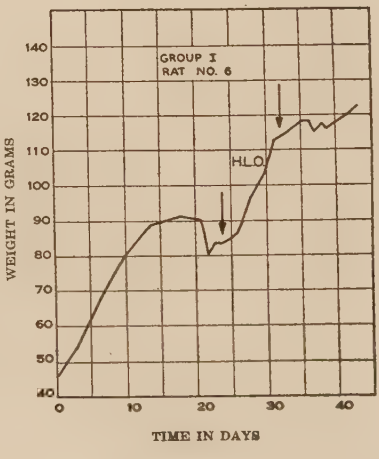


FIG. 6



Space between arrows indicates the interval during which the vitamin was applied.

Growth Curves of Rats to which Halibut-Liver Oil was Topically Applied

FIG. 7

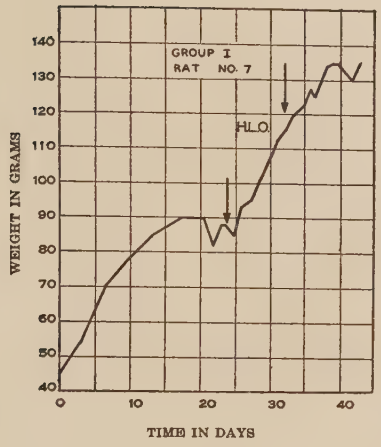


FIG. 8

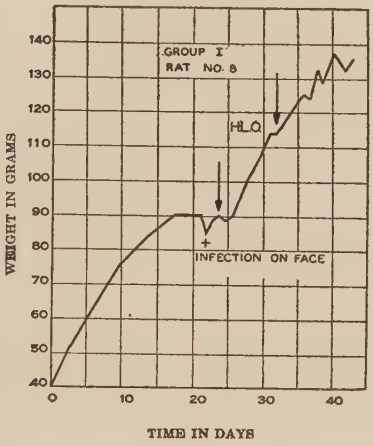


FIG. 9

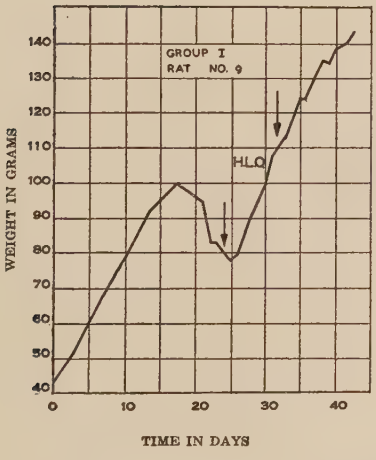
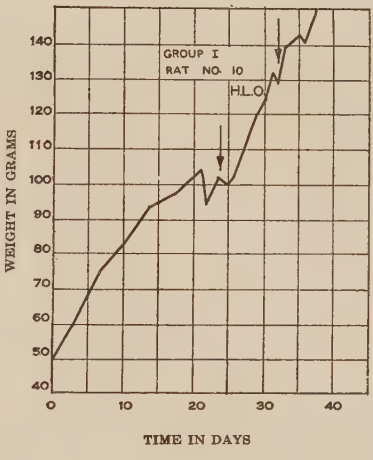


FIG. 10



\+ Very mild xerophthalmia

Space between arrows indicates the interval during which the vitamin was applied.

Growth Curves of Rats used as Negative Controls

FIG. 11

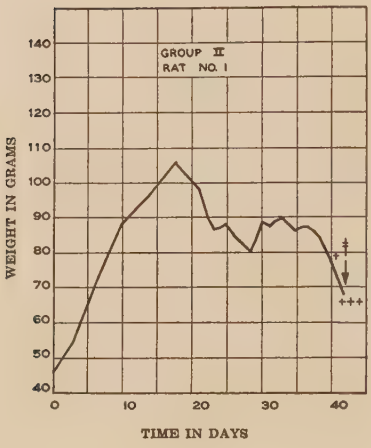


FIG. 12

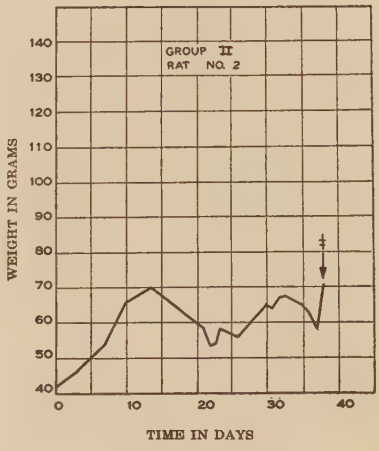


FIG. 13

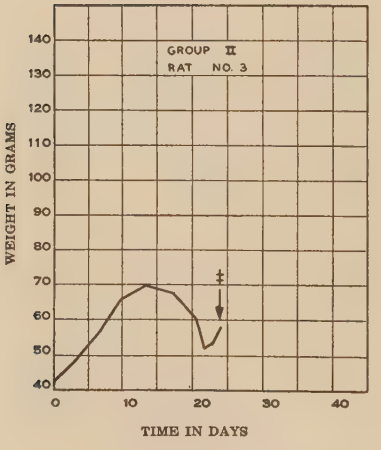
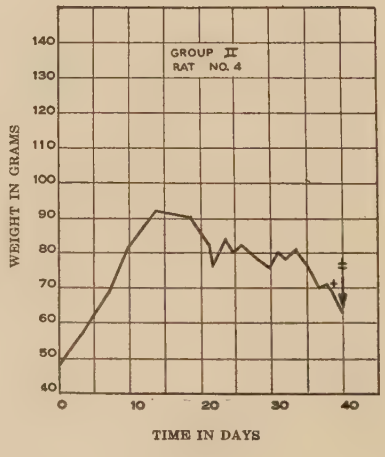


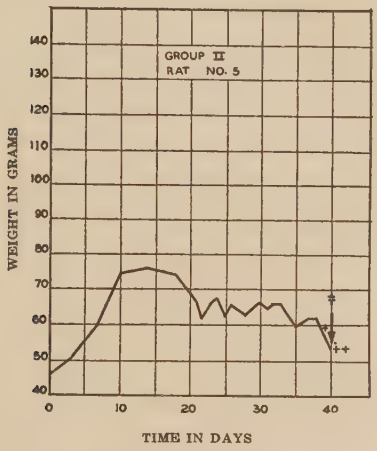
FIG. 14



\+ Very mild xerophthalmia
++ Mild xerophthalmia
+++ Severe xerophthalmia

Growth Curves of Rats used as Negative Controls

FIG. 15



+ Very mild xerophthalmia
++ Mild xerophthalmia

SUMMARY

Ten rats, after a 3-week depletion period on a vitamin A-free diet, were rubbed on the back of the neck with .05 cc. of halibut-liver oil containing approximately 2530 U. S. P. units of vitamin A. The weights of the animals at once increased. Only one rat showed signs of xerophthalmia before treatment began. This rat was cured by the topical applications. In all other cases xerophthalmia was successfully prevented, while severe xerophthalmia developed in the majority of negative controls. The increase in weight of the treated animals continued rapidly even after the application of halibut-liver oil had been discontinued for some time. These experiments indicate that vitamin A, contained in animal oils, is readily absorbed through the skin by topical application.

TOPICAL APPLICATIONS OF VITAMIN A AND OF CAROTENE

Part II—Absorption of Carotene

By AGNES C. HELMER and REV. CORNELIUS H. JANSEN

Simultaneous with the topical applications of vitamin A in halibut-liver oil, parallel experiments were planned to test the absorption of carotene under similar conditions.

The literature contains but few references regarding the topical application of carotene. In 1934 Ratchewsky used a colloidal suspension of an extraction of carotene as a local application in the treatment of injuries to the eye. He states: "The use of carotene is particularly recommended in cases where the fate of the injured area depends on the rapid regeneration of the epithelium and the sub-epithelial nerve endings. . . . The experimental results of the local application of provitamin A agree very well with its general effect on the tissues through oral application."[6]

The work of Frohring affords further proof of the value of carotene to the epithelial layers. "The role of vitamin A in distemper seems to conform to the widely-held conception of the relationship of vitamin A to upper respiratory infections, that vitamin A, by promoting healthy epithelial tissue, particularly of the upper respiratory tract, tends to close this portal of entry to infections." Carotene was used as the source of vitamin A in his experiments.[7]

These conclusions were corroborated by Germann, who states: "Since carotene has as one of its specific functions the protec-

---

[6]Ratchewsky, P. A., *Klin. Wochschr.*, **13** (1934): 918.

[7]Frohring, W. O., *Veterinary Medicine*, **30** (1935).

tion of epithelial tissues in all parts of the body, it is not surprising that carotene has been found of value in such diverse conditions as the following: upper respiratory infections, such as colds; chronic sinus; chronic diarrhea; colitis; kidney and bladder stone; various eye troubles, such as uveitis and conjunctivitis; acne; skin ulcers; pyorrhea and canker sores."[8]

Germann further cites Clausen as believing "that the normal carotene content of human blood is between 80 and 160 micrograms per 100 cc., and that when the carotene content is low, there may be an increased tendency to develop upper respiratory infections."

EXPERIMENTAL

The diet used, the housing conditions and the methods of testing the topically-applied material were the same as those described in Part I.

Less than 0.5 mg. of carotene in oil,* which had a potency of 108,000 U. S. P. units per gram, was applied to the clipped area and rubbed in with the finger until all the oil was absorbed. The amount applied each day was enough to stain the area a deep orange.

At the end of the depletion period the 10 rats in Group III (Figs. 16-25) were clipped, and for 8 days the carotene concentrate was rubbed daily into the skin. Some rats showed a gain in weight 2 days after the treatment began, and all showed a definite gain at the next weighing, 2 days later. This gain in weight steadily continued even after the carotene was no longer applied. At the end of 20 days the gain in weight for each rat in Group III ranged from 35 to 108 per cent,

---

[8]Germann, A. F. O., The Biological Importance of Carotene (An address delivered before the Cleveland Section American Chemical Society, 1935).

*The vitamin concentrate was Smaco Carotene (a product of the S. M. A. Corporation, Cleveland).

the average for the group being 64 per cent. Since the gain in weight continued long after the treatment period had ceased, it seems evident that the absorption, by topical application, of provitamin A (carotene) was not only rapid enough for the animals' immediate needs, but even allowed for the storage of a reserve. The controls were those used in Part I (Group II). None of the rats to which carotene had been applied showed any signs of xerophthalmia, and none of them died during the course of the experiment.

Growth Curves of Rats to which Carotene was Topically Applied

FIG. 16

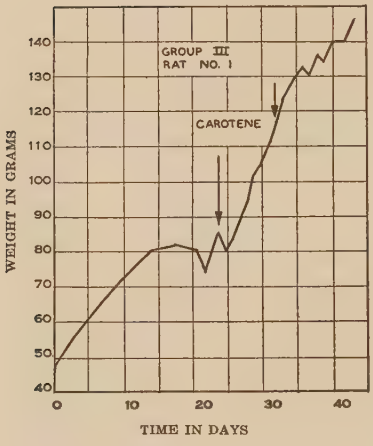
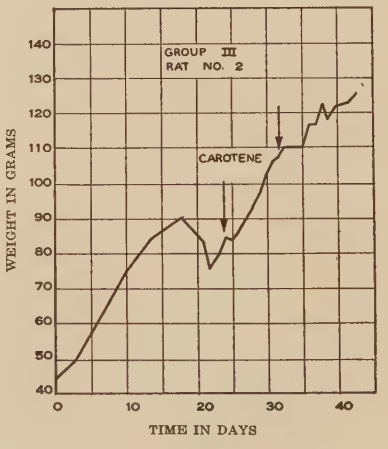


FIG. 17



Space between arrows indicates the interval during which carotene was applied.

Growth Curves of Rats to which Carotene was Topically Applied

FIG. 18

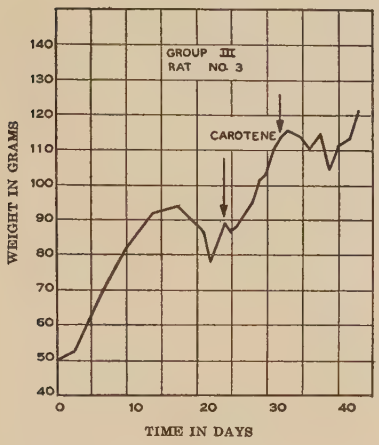


FIG. 19

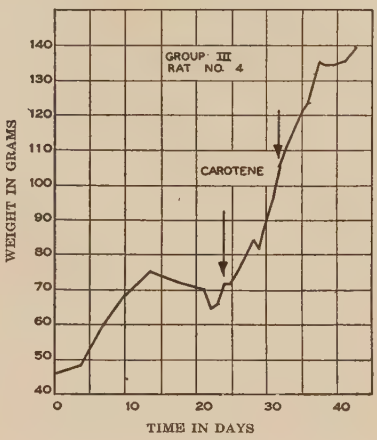


FIG. 20

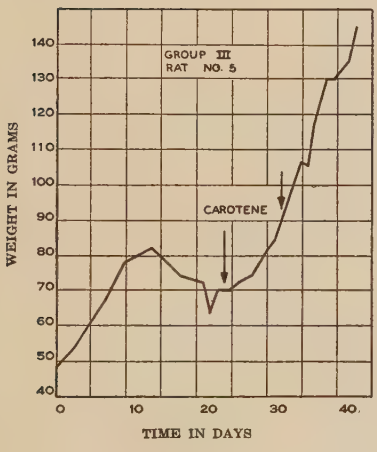
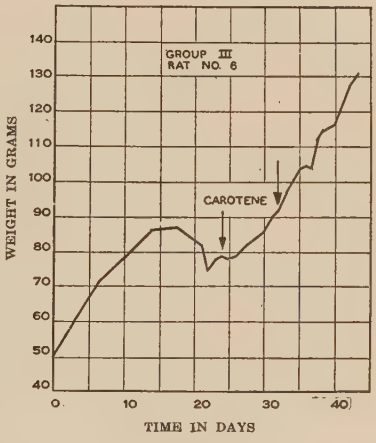


FIG. 21



Space between arrows indicates the interval during which carotene was applied.

Growth Curves of Rats to which Carotene was Topically Applied

FIG. 22

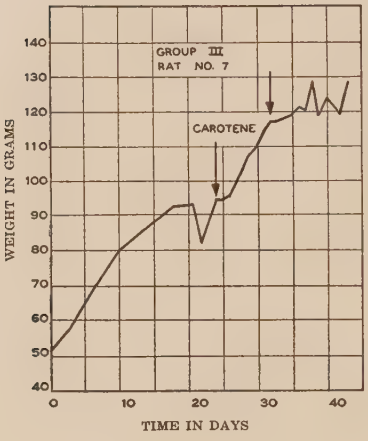


FIG. 23

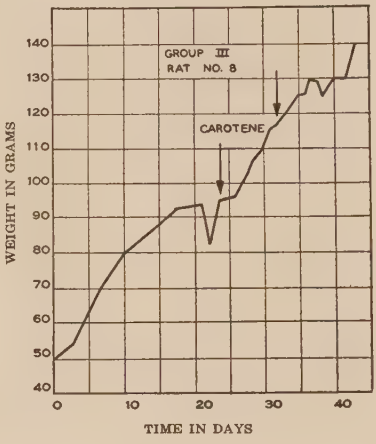


FIG. 24

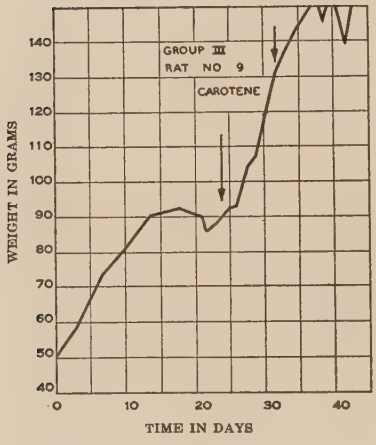
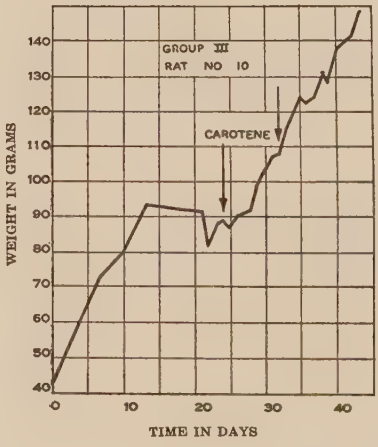


FIG. 25



Space between arrows indicates the interval during which carotene was applied.

SUMMARY

Ten rats, after a 3-week depletion period on a vitamin A-free diet, were rubbed with carotene concentrate on a limited area of skin at the back of the neck. The weights of the rats at once increased and xerophthalmia was prevented in all cases. The increase in weight continued rapidly even after the application of carotene had been discontinued for some time, indicating that vitamin A, in its provitamin form of carotene, is readily absorbed through the skin by topical application.

CONCLUSIONS

Both vitamin A and provitamin A in oil solutions are readily absorbed through the skin when applied topically. They can be absorbed in quantities sufficient to promote growth and to prevent xerophthalmia in a degree comparable with the results produced by their oral administration.

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THE STIMULATION OF YEAST RESPIRATION BY RADIATIONS

By JOHN C. FARDON, SISTER M. JORDAN CARROLL, O.P. and SISTER M. VERONITA RUDDY, O.P.

The possible activation of yeast by irradiation has been the subject of much controversy from the early works of R. de Fazi[1] until the present time. Three types of activation have been considered: *growth*, *fermentation* and *respiration*. Growth and fermentation have been extensively investigated, while respiration has been given only slight consideration and this but in recent years. A perusal of the literature leaves the impression that the results of recent investigations have successfully disproved the existence of the phenomenon of activation by radiations. If the de Fazis were the only investigators to report positive results, the question might be accepted as settled in the negative, but there is no lack of published results of other investigators which support the original observations.[2]

Many refinements in technic have been introduced in an attempt to produce more consistent results. In spite of these precautions it becomes at once apparent, in reviewing the literature, that no standard experimental technic has been adopted. Still more striking is the total disregard of many factors which may play a very decisive part in revealing a stimulation of respiration, fermentation or growth in yeast suspensions treated with ultra-violet radiations.

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[1]de Fazi, R., *Ann. chim. applicata*, 4 (1915): 301.

[2]Lindner, P., *Wochschr. Brau.*, 39 (1922): 166; Owen, W. L., *Food Industries*, 5 (1933): 252; Owen, W. L., and Mobley, R. L., *Zentr. Bakt., Parasitenk.*, 2 (1935): 273.

De Fazi[3] and Lindner,[4] working under simplified conditions, claimed a definite stimulation of fermentation for yeast suspensions treated with ultra-violet radiations. Among later investigators, Tanner[5] irradiated very weak suspensions in distilled water without results. Reynolds and Wynd[6] also reported negative results, but as they admit in their paper, their treatment, as judged by means of the methylene blue test, showed either complete killing or no destructive action upon the cells. Oster[7] studied the problem in detail, using monochromatic light. This procedure was probably dictated by the assumption that there are stimulating and depressing zones in the ultra-violet spectrum. He observed no evidence of a stimulative effect.

Surányi and Vermes[8] studied the stimulation of respiration of yeast by means of ultraviolet, with positive results. However, as will be evidenced from our results, the same lack of consistency was observed in attempts to stimulate respiration by ultraviolet as were encountered in attempts to stimulate growth and fermentation by this agent.

Part I—The Effect of Radiations on the Oxygen Consumption of Yeast

The object of this experiment was to determine the effect of ultra-violet radiations on the respiration of yeast cells in suspension.

A quartz mercury arc was used as a source of radiations. The yeast suspension was placed in a quartz test tube(10cc.

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[3]loc. cit.

[4]loc. cit.

[5]Tanner, F. W., *Chem. Rev.*, **1** (1925): 397.

[6]Wynd, F. L., and Reynolds, E. S., *Ann. Missouri Botan. Garden*, **22** (1935): 771.

[7]Oster, R. H., *J. Gen. Physiol.*, **18** (1934): 71.

[8]Surányi, G., and Vermes, M., *Magyar Orvosi Arch.*, **30** (1929): 585.

capacity) and mounted in front of the arc at a distance of about 10 inches. This distance was chosen because the radiant heat from the lamp kept the suspension at a constant temperature not greatly in excess of 30° C. The yeast suspension was continuously agitated throughout the period of irradiation by an electric stirrer. The stock suspension was prepared by adding one-fourth cake Fleischmann yeast (Saccharomyces cerevisiae) to 200 cc. Ringer glucose phosphate solution, and shaking thoroughly.

For measuring the oxygen consumption, 6 Barcroft-Warburg manometers were used. It was found necessary to enclose the manometers and the water-bath in a constant-temperature chamber. This chamber consisted of a large wooden box with a glass front which made it possible to read the manometers without opening the chamber. A row of small openings opposite the adjusting screws made it possible to adjust the manometers from without. The box was maintained at a temperature of approximately 35° C. The manometers under these new conditions showed variations not in excess of 4 per cent.

As a rule 3 cc. of the control or experimental suspension were introduced into the outer vessel of the manometer flask and 0.3 cc. of a 5 per cent potassium hydroxide solution was pipetted into the inner well.

About 7 cc. of the stock yeast suspension were irradiated under the ultra-violet arc for a period of 5 min. The manometer flasks were then charged as follows:*

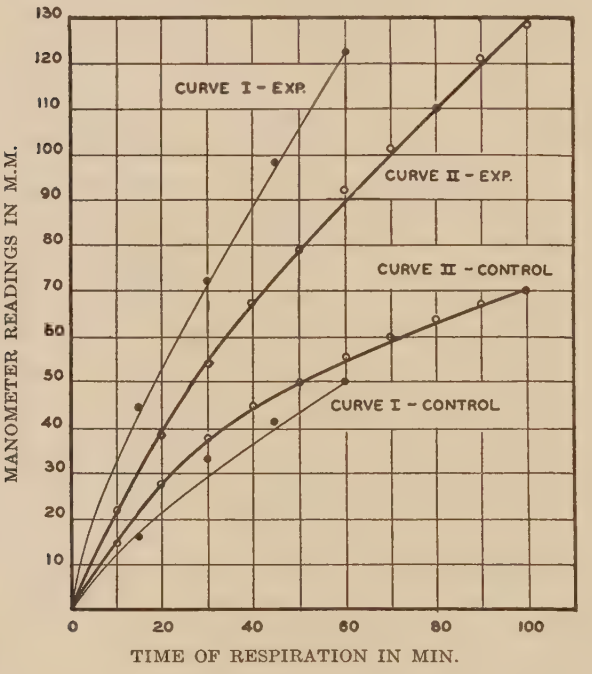
Control . . . . . 2 cc. stock suspension plus
1 cc. Ringer solution

Experimental . . 2 cc. irradiated suspension plus
1 cc. Ringer solution

*Throughout these experiments the practice was to use 3 flasks for the controls and 3 for the experimentals.

The respirometers were then operated for a period of 60 min. As can be observed from Fig. 1, Curve I, the experimental suspensions consumed over 100 per cent more oxygen than did the control suspensions.

FIG. 1—*The Effect of Ultra-Violet on the Oxygen Consumption of Yeast*



The control and experimental suspensions were then taken out of the flasks and tested with a solution of methylene blue to determine the percentage of living yeast cells present. Microscopic examination revealed a greater percentage of living cells in the control than in the experimental suspensions.

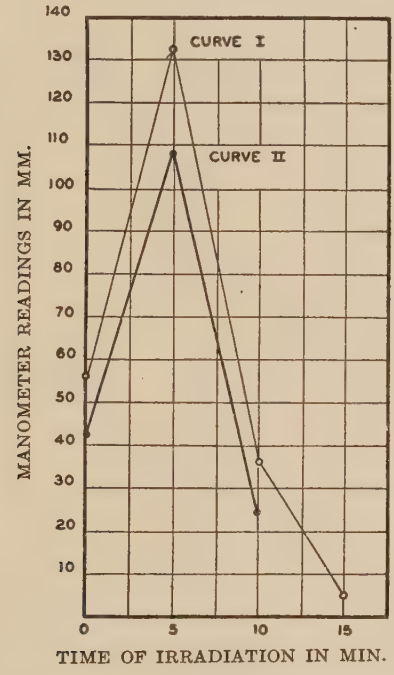
The experiment was then repeated under identical conditions, except that the respiration time was extended to 100 min. The results were about the same (Fig. 1, Curve II).

Optimum Exposure for Stimulation of Respiration

This phase of the research was to determine the time of exposure required for maximum stimulation of respiration.

Five equal portions of the stock yeast suspension were measured and irradiated for periods ranging from 5 to 25 min., each period 5 min. longer than the preceding. As in the previous experiment, the controls were made up of 2 cc. of the stock suspension and 1 cc. Ringer solution, and the experimentals were made up of 2 cc. irradiated suspension and 1 cc. Ringer

FIG. 2—*Optimum Exposure for Respiratory Stimulation*



solution. The respiration was measured over a period of 40 min. Maximum oxygen consumption occurred in the suspension which had been irradiated for 5 min. As shown in the curves of Fig. 2, the respiration dropped rapidly after 5 min. The

experiment was repeated the following day (Curve II) and, although the oxygen consumption was not as great in both control and experimental as was previously obtained (Curve I), maximum stimulation again occurred at 5 min. Since only one reading was taken between the 0 and the 10-minute interval, the 5-minute interval cannot be relied upon as the exact time for maximum respiration.

Correlation of Respiration with Growth

In this experiment an attempt was made to correlate the oxygen consumption of the irradiated yeast suspension with the number of yeast colonies grown on Difco nutrient agar.

Five equal portions of stock yeast suspension were measured and irradiated from 1 to 5 min. respectively. After irradiation, a measured amount was taken from each sample, diluted and plated out on petri dishes for incubation. At the same time portions of these samples were tested in the respirometers.

TABLE I

The relation of growth and respiration to time of irradiation

Time of Irradiation	Increase in Respiration	Number of Colonies after 24 Hours' Incubation
0 minutes	Control	500
1 minute	10 per cent	9
2 minutes	34 per cent	0
4 minutes	49 per cent	0
5 minutes	81 per cent	0

The results of the experiment, given in Table I, show that an increase in respiration is not accompanied by an increase in cell growth. Not only was there no evidence of growth stimulation accompanying the stimulation of respiration, but at the end of 24 hr. the petri dishes showed that no colonies

had developed from suspensions irradiated 2 min. or longer. Forty-eight hours later a growth of approximately 500 yeast colonies per dish was obtained in some of the petri dishes, which had shown no evidence of growth after the first 24 hr., indicating that the cells had not been killed but that their growth rate had been retarded. When these experiments were duplicated, stimulation of respiration did not always occur. This suggested that other factors might exert an influence on respiration. An investigation of these factors is reported in another paper.*

Respiration as a Function of Age of Yeast Suspension

This experiment was conducted to determine the effect of the age of the stock yeast suspension on respiration after irradiation. The respiration of all samples was measured over a period of 40 min.

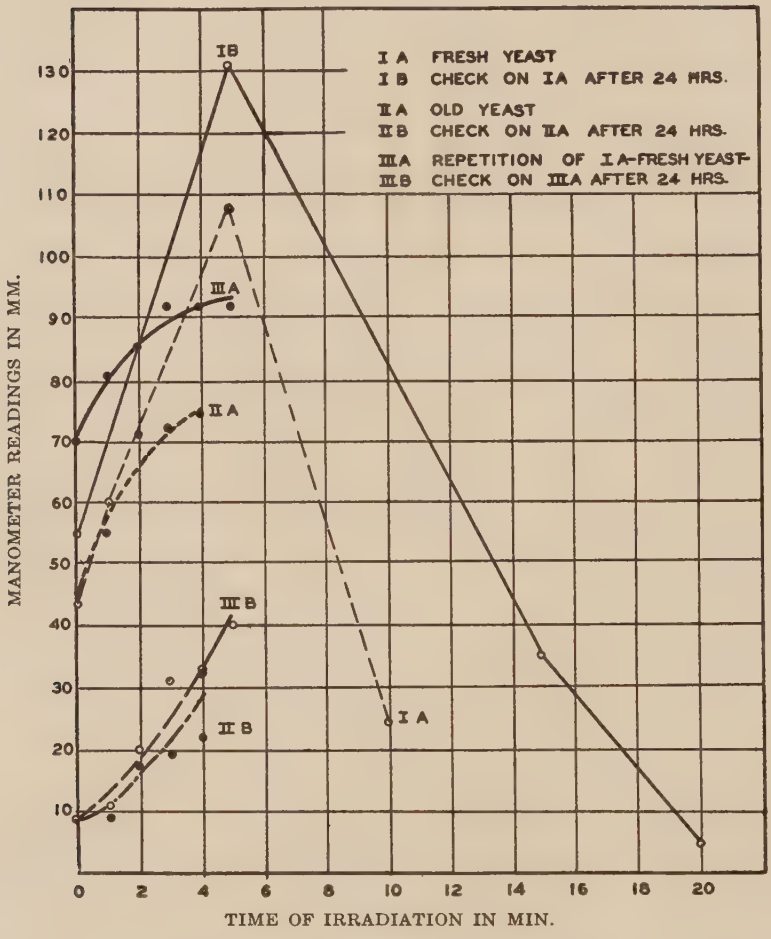
The first case was a suspension prepared from a fresh yeast cake. The respirometer flasks were charged in the usual manner, viz., 2 cc. stock suspension and 1 cc. Ringer solution for the experimental. The samples were irradiated for various intervals, ranging from 1 to 20 min. The maximum oxygen consumption again occurred after 5 min. of irradiation, giving an increase of 157 per cent.

The experiment was repeated the following day, using the same stock suspension under identical conditions. The peak remained at 5 min. and the increase in respiration was found to be 141 per cent, which checked with the result obtained the previous day. In this experiment, however, the oxygen consumption of both control and experimental was slightly above that of the previous one.

An old stock suspension which had been standing in the refrigerator for several weeks was then tested. Since the

*Norris, R. J., and Ruddy, Sr. M. Veronita, p. 53, this issue.

FIG. 3—*Respiration as a Function of Age of the Yeast Suspension*



manometer readings were of such magnitude in the two previous experiments as to utilize nearly the entire manometer scale, only one-half of the suspension was introduced into the flasks, i.e., both control and experimental flasks were charged with 1 cc. stock suspension and 2 cc. Ringer solution. A

maximum stimulation of 71 per cent was obtained at the 5-minute period of irradiation.

In order to determine whether this stimulation was a temporary effect or whether it persisted, the suspensions were allowed to remain in the respirometers until the following day when their oxygen consumption was again determined (Curve II-B). Although the total oxygen consumption dropped considerably, a stimulation over the control of 265 per cent was obtained, indicating that this stimulating effect continues for at least 24 hrs. after the cells have been subjected to irradiation.

An experiment was then conducted with a newly-prepared stock yeast suspension. The amount of material introduced into the flasks was the same as in the preceding experiment. An increase in respiration of 35 per cent was obtained (Curve III-A). When the experiment was repeated the following day, an increase of 357 per cent was obtained, though again the total oxygen consumption dropped considerably (Curve III-B).

In Table II are recorded the stimulation of respiration, together with the respiration of the control.

Depression of Respiration as a Result of Irradiation

In view of the fact that the stimulation of respiration by irradiation was not consistent, the following group of experiments was conducted in an attempt to determine the variables responsible for the differences found.

The same technical procedure was used, with the exception that the respiration time was decreased to 25 min. The result was entirely unexpected; there was no evidence of stimulation after irradiation, but rather a marked decrease in oxygen consumption as shown in Fig. 4, Curve I. The experiment was repeated, but again a definite depression was noted, as shown in Fig. 4, Curve II. Another fresh stock yeast suspension was

TABLE II

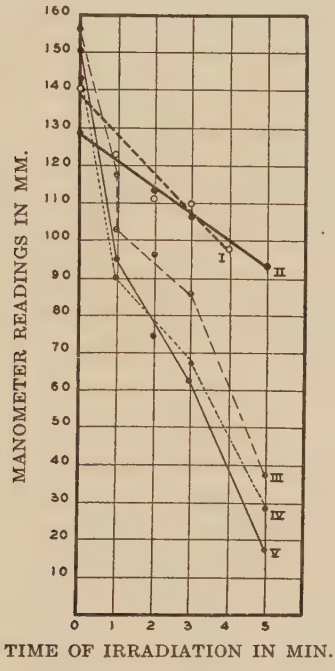
Stimulation of Respiration by Ultra-violet Irradiation as a Function of the Age of the Yeast

Age of Suspension	Respiration Period	Time of Irradiation in minutes	Contents of Manometers	Cu. mm.* Oxygen Uptake Control	Cu. mm.* Oxygen Uptake Experimental	Per Cent Increase Oxygen Uptake
Fresh	40	5	2 cc. yeast suspension 1 cc. Ringer sol.	57.40	147.85	157
After 24 hrs.	40	5	"	74.76	180.71	141
2 weeks	60	5	1 cc. yeast suspension 2 cc. Ringer sol.	60.08	103.05	71
After 24 hrs.	60	5	"	12.02	43.96	265
Fresh	60	5	"	93.45	126.43	35
After 24 hrs.	60	5	"	12.02	54.96	357

*These data are computed from manometer readings recorded in Fig. 3.

then prepared and the experiment repeated several times during the course of the day. In every case depression of oxygen consumption was observed, Curves III, IV and V practically falling upon each other.

FIG. 4—*Depression of Respiration as a Result of Irradiation*



A study of the rate of destruction of yeast cells as a function of time shows that at the end of 5 min. a large percentage of the yeast cells had been destroyed. An analysis of the respiration data, based on the number of living cells rather than the total number of cells, showed in each case that although the total oxygen consumption of the yeast suspension was below the control at the end of 5 min., the oxygen consumption per living cell had increased, i.e., the remaining living cells showed greater oxygen consumption than the cells of the control.

Some time later another fresh suspension was prepared and stimulation again obtained after irradiation. In one instance 31 per cent stimulation was observed and in another 240 per cent.

The Effect of pH on the Respiration of Irradiated Yeast Suspensions

It was quite evident by this time that some unknown variable factor was responsible for the inconsistent results obtained in the stimulation and depression of oxygen consumption. It was thought that the respiration of yeast cells might be affected by changes in pH and that the yeast suspensions standing in the laboratory for various periods of time might experience a change in hydrogen ion concentration. To determine the effect of changes in this factor, 6 yeast suspensions were prepared which were identical except for pH values. These values and the resultant readings are given in Table III.

TABLE III

Effect of pH on the Stimulation of Yeast Respiration by Ultra-violet Irradiation

Respirometer Number	pH of Yeast Suspension	Manometer Reading after 20 Minutes
1	6.9	66
2	7.1	66
3	7.3	75
4	7.5	67
5	7.7	69
6	7.8	61

It is evident from these results that although an optimum pH value may exist, the hydrogen ion concentration, even in such wide variations as those employed (6.9 to 7.8), does not materially alter the oxygen consumption.

The Effect of Radiations on the Respiration of Actively Budding Yeast Cells in Suspension

The object of the experiment was to determine whether actively budding yeast cells were required in order to obtain stimulation after irradiation. A pure strain of yeast (Saccharomyces cerevisiae) was grown in raisin culture fluid which was prepared from raisin extract plus 0.1 per cent yeast ash, under sterile conditions. The culture was inoculated with a colony of yeast cells which had been grown on nutrient agar and then incubated for several days. The yeast suspension was centrifuged, washed several times in Ringer solution and irradiated, and the flasks charged in the usual manner. The irradiated suspension gave a depression of oxygen consumption, dropping to a value as low as 40 per cent below that of the control. This indicated that the rate of growth of yeast cells is not of prime importance in the stimulation of respiration by irradiation.

The Effect of Storage Temperature on the Respiration of Irradiated Yeast Suspensions

During the course of the experiments the stock yeast suspensions were not always kept under the same conditions; sometimes the suspensions were stored in the refrigerator and at other times they were allowed to stand at room temperature. It was thought that this might in some way alter the yeast and as a consequence affect the respiration of the suspension. To test this, a fresh yeast suspension was prepared and divided into 3 portions: one portion was kept in the refrigerator at 10° C.; another at room temperature, 21° C.; and the third portion in the incubator at 37° C.

The suspensions were then divided into control and experimental portions. The experimental groups were irradiated for 5 min. The respiration was measured over a period of 40

min. The sample which had been kept overnight at 10° C. showed a drop in oxygen consumption of 37 per cent; the sample kept at 21° C. suffered a depression of 40 per cent; while the sample at 37° C. dropped 18 per cent below the control value. Whether or not the variations from 18 to 40 per cent are significant cannot be stated without repeating the experiments. For this experiment suffice it to say that no stimulation was obtained under these conditions.

Influence of Selected Wavelengths on Respiration

Ultraviolet—In all the later experiments the depressive effect of irradiation was so consistent that it was thought some change might have taken place in the output of the ultra-violet lamp: the voltage across the arc might have fluctuated, or a film of grease might have been burned off the quartz element. Either change would result in a difference in output, especially in the shorter region of the spectrum.

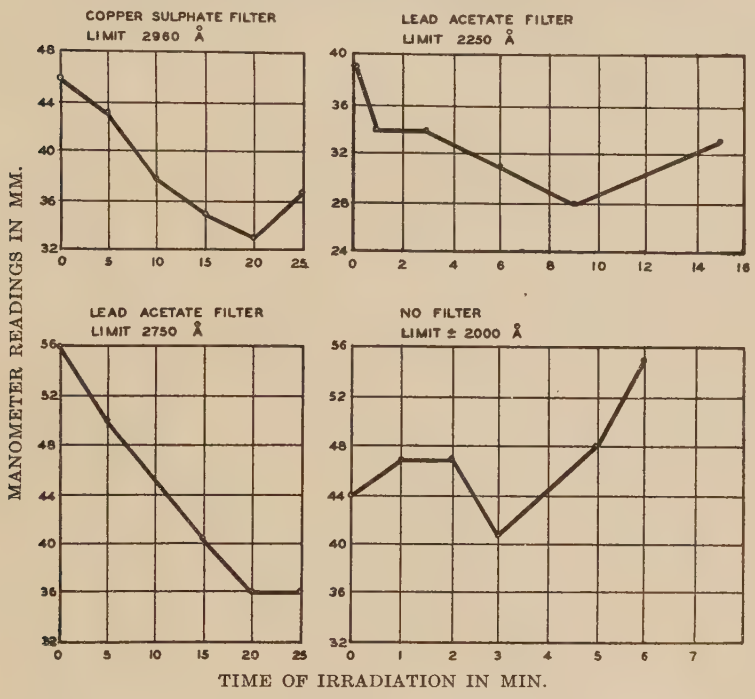
In an attempt to establish the possible influence of the shorter wavelengths on respiration, quantities of stock yeast suspension were prepared in the usual manner and treated with four different spectral bands. The different spectral bands were secured through the use of ultra-violet absorbing filters:[9] one a copper sulphate filter with an absorption limit of 2960 Å; one a lead acetate filter with a limit of 2750 Å; the third, a lead acetate filter, with a limit at 2250 Å; the fourth, an unfiltered quartz mercury arc.

A yeast suspension was irradiated through the copper sulphate filter and after 5 min. a portion was removed and tested in the manometers. Other portions were removed after each 5-minute interval, the last having been irradiated 25 min. There was a depression of respiration noted in each sample taken, the depression increasing as the time of irradiation

[9]Schneider, H., and Sperti, G., The Quantum Theory in Biology, (1926).

increased excepting the sample irradiated 25 min., which showed a slight decrease over the 15 and 20-minute samples. (Fig. 5.)

FIG. 5—*Effect of Selected Wavelengths on Respiration*



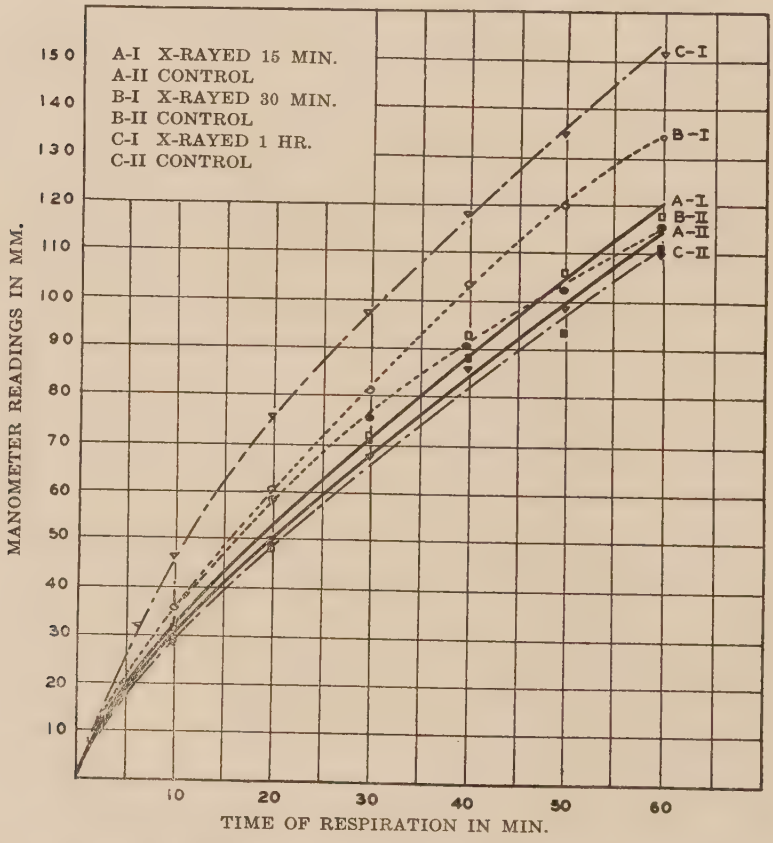
Similar portions were also irradiated through the lead acetate filter with an absorption limit of 2750 Å. Portions were removed at the 5, 15, 20 and 25-minute intervals. These gave a decrease in respiration with increase in time of irradiation for all samples except the one which had been irradiated 25 min. (Fig. 5.)

Next, a yeast suspension was irradiated with the lead acetate filter with an absorption limit of 2250 Å. Portions were removed at the 1, 3, 6, 9 and 15-minute intervals. Again there was a decrease of oxygen consumption, this decrease continuing with

the time of irradiation with exception of the last reading, which had increased above both the 6 and the 9-minute interval. (Fig. 5.)

A fourth suspension was irradiated without filter with an irradiation limit at approximately 2000 Å. Portions were removed after 1, 2, 3, 5 and 6 min. All samples showed an increase in respiration excepting the one removed after 3 min. (Fig. 5)

FIG. 6—*Effect of X-Rays on the Respiration of Yeast*



These experiments indicate that although wavelength might play some part in the stimulation of respiration of yeast cells

by irradiation, it could by no means be considered a factor of prime importance in our experiments.

X-rays—A stock yeast suspension was prepared in a manner similar to that used in the ultra-violet experiments. About 20 cc. of the suspension were pipetted into a petri dish and placed under a Coolidge tube at a distance of 12 in. from the target. The voltage was kept at 45 kv. and the current at 10 Ma. The yeast suspension was irradiated for 15 min. Three respirometer flasks were each charged with 3 cc. stock suspension and 3 were charged with 3 cc. irradiated suspension. Readings were taken at 10-minute intervals over a period of 1 hr. Curves A-I and A-II, Fig. 6, in which the respirometer readings are plotted, show an increase of about 5 per cent in oxygen consumption for the irradiated suspensions.

The time of irradiation was then increased from 15 to 30 min., all other conditions being the same. A stimulation of about 20 per cent was obtained over a respiratory period of 1 hr. (Curves B-I and B-II.)

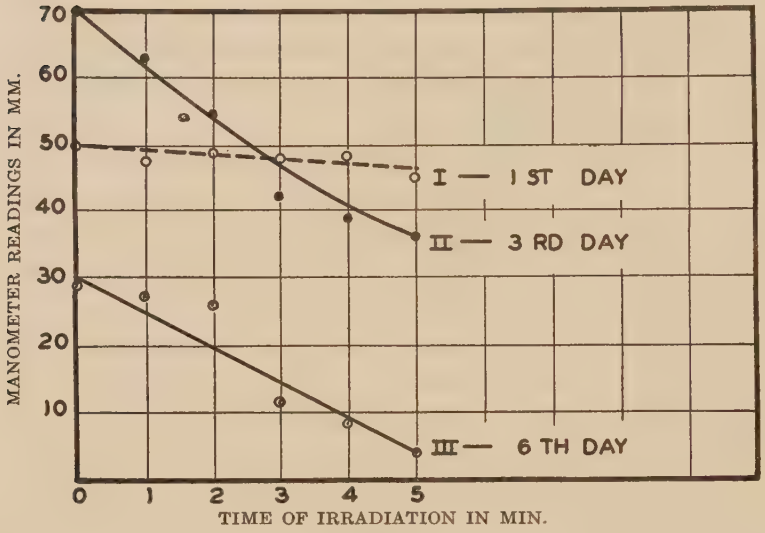
The time of irradiation was now extended to 1 hr., resulting in a maximum stimulation of 35 per cent in a respiratory period of 1 hr. (Curves C-I and C-II.)

Respiration as a Function of Bacterial Contamination

This experiment was conducted to determine whether bacterial contamination of the yeast suspension was the cause of the marked stimulation obtained in the earlier experiments.

A yeast suspension was kept unstoppered in the incubator overnight. A microscopic examination on the following morning of a drop of the suspension revealed the presence of a number of bacteria in the preparation. The experimental groups were irradiated for 5 min. and the suspensions were then tested in the respirometers. The respiration was measured over a period of 25 min. and a drop of 12 per cent in respiration noted. Three days later a portion of the same stock suspension

FIG. 7—*Respiration of Irradiated Yeast as a Function of Bacterial Contamination*



was tested and a drop of 47 per cent obtained. A portion of this same sample, tested 6 days later, showed a reduction of 85 per cent (Curves I, II and III, Fig. 7). Although these results seem to indicate that incubation (probably contamination) has some influence on the depression of respiration, in no case was stimulation noted.

SUMMARY

1. The stimulation of the respiration of yeast suspensions resulted from the direct irradiation of cells by ultra-violet and X-rays.

2. In all cases it was found that the respiration of the remaining living cells was stimulated by irradiation.

3. An optimum exposure exists for maximum stimulation.

4. Growth, pH, bacterial contamination, age of culture and storage temperature had little effect on the stimulation of respiration induced by ultra-violet radiations.

THE STIMULATION OF YEAST RESPIRATION BY RADIATIONS

Part II—The Influence of Killed Cells on the Respiration of Living Cells

By JOHN C. FARDON, SISTER M. JORDAN CARROLL, O.P. and SISTER M. VERONITA RUDDY, O.P.

As a result of an analysis of the respiration-time curves it became apparent that two factors were influencing respiration: one tending to increase oxygen consumption, the other tending to reduce it. An experiment soon verified the fact that when the yeast cells in the suspension had all been killed by ultra-violet light, they did not consume oxygen. Clearly, then, the killing of yeast cells was a major factor in the reduction of respiration which in many cases followed irradiation.

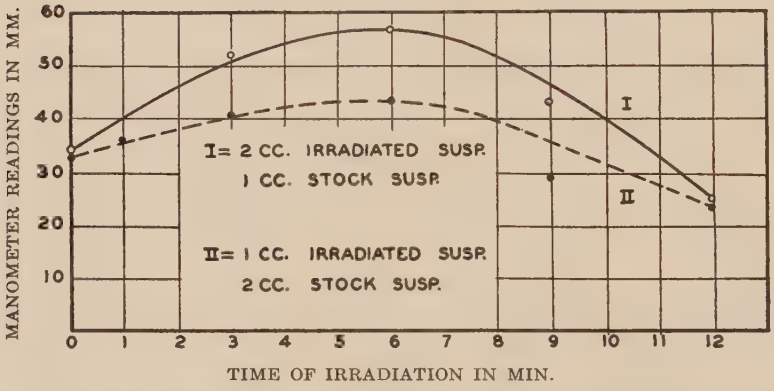
It was thought possible that in the irradiation of the yeast suspensions too many of the yeast cells were destroyed and the stimulative action induced was altogether overshadowed by this condition. Experiments were planned in which the irradiated yeast suspensions were added to non-irradiated stock suspensions to determine the soundness of this theory.

The Effect of the Addition of Irradiated Yeast Suspensions on the Respiration of Non-Irradiated Suspensions

A stock yeast suspension was prepared in the usual manner. The control flasks were charged with 3 cc. stock suspension. In the experimental series, in one group each flask was charged with 1 cc. irradiated suspension plus 2 cc. stock suspension; in another group each flask received 2 cc. irradiated suspension and 1 cc. stock suspension. The experiment was repeated five

times, the irradiation period only having been changed. Although stimulation of respiration was not as marked as in the earlier cases in which the respiration of the irradiated suspension itself was studied, definite stimulation was obtained, showing a maximum after 5 min. of irradiation (Curves I and II, Fig. 8).

FIG. 8—*Effect of Irradiated Suspensions on Respiration of Non-Irradiated Suspensions*



Further study is necessary to determine definitely if continued irradiation destroys the respiratory stimulating factor.

The Effect of the Addition of Heat-Treated Yeast Suspensions on the Respiration of Stock Suspensions

This experiment was conducted to determine whether the stimulative effect obtained through irradiation could also be obtained by subjecting the yeast suspension to heat.

A portion of the stock suspension was kept at room temperature as control; another was exposed to a temperature of 100° C. for 15 min.; a third portion was exposed to a temperature of 60° C. for 15 min.

The manometer flasks were then charged as follows:

Flasks Nos. 1 and 2..2 cc. stock suspension plus
1 cc. Ringer solution

Flasks Nos. 3 and 4..2 cc. stock suspension plus
1 cc. suspension exposed to 100° C.

Flasks Nos. 5 and 6..2 cc. stock suspension plus
1 cc. suspension exposed to 60° C.

Respiration was measured over a period of 25 min. with the following results:

Control..37 cu. mm. oxygen consumption
100° C...36 cu. mm. oxygen consumption
60° C...41 cu. mm. oxygen consumption

Since no determination was made of the number of surviving cells after subjecting them to the respective heat treatments, it could not be stated whether the 60° C. sample actually showed a stimulation in respiration or whether this apparent increase in oxygen consumption was due to the addition of cells which had not been completely destroyed by the heat.

Since these results were inconclusive it was decided to repeat the experiments with slight modifications.

The Effect of the Addition of Autoclaved Yeast Suspensions on the Respiration of Stock Suspensions

A stock suspension was divided into two equal portions, one of which was kept at room temperature as control, the other autoclaved for 15 min. at 20 lb. pressure. The control manometer flasks were charged with 0.5 cc. of the control suspension and 2.5 cc. Ringer solution. The experimental flasks were charged with 0.5 cc. of the control suspension, 0.5 cc. of the

autoclaved suspension and 2.0 cc. Ringer solution. Respiration was measured over a period of 25 min. with the following results:

Control . . . . . 46.2 cu. mm. oxygen consumption
44.3 cu. mm. oxygen consumption
45.2 cu. mm. (average)

Experimental . . 61.6 cu. mm. oxygen consumption
59.3 cu. mm. oxygen consumption
60.4 cu. mm. (average)

Stimulation: approximately 33 per cent.

These results show that the stimulative factor can also be produced by subjecting the yeast suspension to heat.

SUMMARY

The addition of cells, killed either by prolonged ultra-violet irradiation or by autoclaving, produces definite stimulation of respiration when added to fresh yeast suspensions.

CONCLUSIONS

The respiration of yeast cells can be decidedly stimulated by ultra-violet and X-ray irradiation. However, the fact that this stimulation can be obtained not only in the irradiated suspensions themselves, but also in non-irradiated suspensions when irradiated or heat-treated cells are added, indicates that the increase in respiration is not the result of the direct action of the radiations. It is caused rather by a fraction which is produced from cells injured or destroyed by the heat treatment or irradiation.

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A RESPIRATORY STIMULATING FACTOR

By JOHN C. FARDON and SISTER M. VERONITA RUDDY, O.P.

In earlier investigations of the effect of irradiated yeast suspensions on non-irradiated yeast suspensions,* there was observed a stimulative effect on the respiration of the untreated yeast. This led to the assumption that the yeast cells, either injured or destroyed by the radiations, liberated a product which caused increased respiration of normal living cells.

In order to investigate the nature of this stimulative fraction, a series of preliminary experiments was conducted.

EXPERIMENTAL

In these experiments 3 Barcroft-Warburg respirometers were used for control and 3 for the experimental yeast suspensions.

The stock yeast suspensions were prepared by placing one-fourth cake of Fleischmann yeast (Saccharomyces cerevisiae) in 200 cc. of Ringer glucose phosphate solution and shaking thoroughly to insure uniform distribution of the yeast cells.

Unless otherwise stated, the experimental yeast suspensions were irradiated for 20 min. with full ultra-violet from a quartz mercury arc, at a distance of 10 inches and constantly stirred in a ½-inch quartz test tube. This time of irradiation was chosen because it produced complete killing of the cells, as determined by both the methylene blue test and the plating-out method on culture media.

Separation of the Stimulating Factor

The first step in the isolation of the stimulating fraction was to separate the cells from the Ringer solution in which they

*Fardon, J. C., Carroll, Sr. M. Jordan, O.P., and Ruddy, Sr. M. Veronita, O.P., p. 17 this issue.

had been irradiated and to determine the relative effect of the two components on the respiration of a stock yeast suspension.

After the suspension had been irradiated for 20 min. to insure complete killing of all yeast cells, it was centrifuged for 15 min. to separate the cells from the Ringer solution. The Ringer solution was then carefully pipetted from the centrifuge tube. The cells were washed, recentrifuged and then suspended in an amount of Ringer solution equal to that removed originally.

The respirometer flasks were charged as follows:

Control .... ½ cc. stock yeast suspension plus
2½ cc. Ringer solution

Sample A .. ½ cc. stock yeast suspension plus
½ cc. pipetted fluid plus
2 cc. Ringer solution

Sample B .. ½ cc. stock yeast suspension plus
½ cc. dead cells suspended in Ringer solution plus
2 cc. Ringer solution

The respiration was measured over a period of 40 min. Sample A gave an increase in oxygen consumption of 40 per cent over that of the control, while Sample B gave an increase of only 10 per cent over the control.

To make certain that Sample A contained no cells, it was passed through a Seitz bacterial filter and the experiment repeated. The result was found to be practically the same as with the centrifuged fraction.

It is apparent from these tests that the major portion of the stimulative factor is present in the cell-free fraction.

Does the Cell-Free Fraction Consume Oxygen?

This experiment was conducted to determine if the centrifuged fluid itself, obtained from the irradiated suspension, consumed oxygen.

Six respirometers were used and each flask was filled with 3 cc. of the fluid. After a 30-minute run it was found that no oxygen had been taken up by the fluid.

For all practical purposes the cell-free fraction need not be considered as playing any direct rôle in the oxygen uptake in respiration experiments. Its action is solely that of stimulating the activity of the living cells.

Stability of the Stimulative Factor to Ultra-Violet Rays

The question arose whether the cell-free fraction could be rendered inactive by prolonged exposure to the unfiltered ultra-violet rays of a quartz mercury arc. To determine this, the irradiation time was doubled. The procedure was the same as that in the first experiment.

It was found that after 40 min. of irradiation the fraction still retained its stimulative effect upon the stock suspension. There was an increase of 38 per cent in oxygen consumption over the control, which is practically the same as that obtained with 20 min. of irradiation. This experiment seems to indicate that under these conditions an irradiation time of 40 min. is not excessive.

Stability of the Stimulative Fraction with Time

A fresh stock suspension was prepared and irradiated for 20 min., centrifuged for 15 min. and the fluid drawn off with pipettes and stored in the refrigerator.

The results of a study of the stability of the respiratory fluid on standing are reported in Table I.

TABLE I

Storage Time	Stimulation of Oxygen Consumption
21 hrs.	40 per cent
45 "	44 " "
90 "	46 " "
117 "	41 " "

Since the variations in stimulative power given in the Table are well within experimental error, it may be concluded that the activity of the fraction was apparently unimpaired after standing for a period of 117 hrs.

For experimental purposes the fraction may be considered extremely stable and may be stored with safety in a refrigerator for at least 100 hrs.*

Strength of Stimulative Fraction as Function of Age of Yeast on which Fraction is Tested

To determine whether or not the strength of the stimulative fraction varies with the age of the yeast on which it is tested, it was decided to compare the stimulative action of a given fraction on a new and on an old yeast suspension.

A fresh stock suspension of yeast was irradiated and a portion of the centrifuged fluid added to a non-irradiated suspension of freshly prepared yeast. The irradiated fraction increased the oxygen consumption of the fresh yeast suspension over that of the control by approximately 53 per cent. A portion of the centrifuged fluid which was tested on fresh yeast was then tested on a suspension of non-irradiated yeast cells which had been stored in the refrigerator for 5 days. The result was a 33 per cent increase in oxygen consumption over the control. The experiments indicate that the age of the yeast suspension may determine to some degree the stimulating power of a given fraction. The variation, however, is not great enough to have an important bearing on the experiments herein presented.

*In a later experiment it was shown that after 4 months of storage in the refrigerator no loss in potency resulted.

Effect of Cell-Free Fluid, Irradiated After Extraction, on Non-Irradiated Yeast Suspensions

Earlier investigators[1] had found that glucose could be changed by ultra-violet light. Wynd and Reynolds refer to a paper by de Fazi (1927) in which he describes the effect of the ultra-violet irradiation of glucose solutions before inoculation with Saccharomyces cerevisiae. Although the yeast itself was not irradiated, he found an increase in fermentation.

The object of this experiment was to determine whether the yeast cells themselves had to be acted upon directly by irradiation in order to produce the respiratory stimulating fraction, or whether the radiations acting only upon the solution in which the cells are suspended was sufficient to produce the activation.

A stock yeast suspension was centrifuged for 15 min. and the fluid pipetted off and irradiated for a period of 20 min. The respirometer flasks were then charged as follows:

Control.....	½ cc. stock yeast suspension plus 2½ cc. Ringer solution
Experimental..	½ cc. stock yeast suspension plus 1 cc. Ringer solution plus 1½ cc. irradiated fluid

The respiration was measured over a period of 40 min. and the oxygen consumption found to be 78 per cent greater than that of the control. A part of this stimulation was probably due to the effect of ultra-violet light on the glucose in the Ringer solution as is indicated by the experiments which follow. Some of the stimulative fraction may also have been produced by the yeast cells before their removal.

The Effect of Irradiated Ringer Solution on the Respiration of Yeast

In view of the results obtained with the irradiated fluid, it was decided to irradiate the Ringer solution alone and determine its effect upon the respiration of a stock yeast suspension.

[1]Wynd, F. L., and Reynolds, E. S., *Ann. Missouri Botan. Garden*, **22** (1935): 771. Also unpublished results of R. J. Norris of this laboratory.

A quantity of Ringer solution containing .02 per cent glucose was irradiated for 20 min. and the respirometers were charged as follows:

Control..... ½ cc. stock yeast suspension plus
2½ cc. Ringer solution

Experimental.. ½ cc. stock yeast suspension plus
1 cc. Ringer solution plus
1½ cc. irradiated Ringer solution

The respiration was measured over a period of 40 min. and the experimental showed a 14 per cent increase of oxygen consumption over that of the control. This value is considerably greater than experimental error and therefore is significant in showing that the Ringer solution can be activated by ultra-violet light. These results were confirmed by later experiments.

The Effect of Irradiated Ringer Solution Minus Glucose

Can stimulation be obtained by irradiating the Ringer solution without the glucose? To determine this the respirometers were charged as follows:

Control..... ½ cc. stock yeast suspension plus
2½ cc. non-irradiated Ringer solution containing glucose

Experimental.. ½ cc. stock yeast suspension plus
1 cc. non-irradiated Ringer solution containing glucose
1½ cc. irradiated Ringer solution minus glucose

After a 40-minute respiration period, the experimental showed only a 3 per cent increase in oxygen consumption over the control. Since this 3 per cent difference lies well within experimental error, it could not be safely stated that the irradiation of Ringer solution minus glucose resulted in a stimulation of oxygen consumption of yeast cells.

Effect of Irradiated Glucose Added to Non-Irradiated Ringer Solution

The following experiment was conducted to determine whether a solution of irradiated glucose when added to Ringer solution would produce an increase in the respiration of a stock yeast suspension.

A 10 per cent glucose solution was irradiated for 20 min. Of this irradiated solution 1 cc. was added to 500 cc. Ringer solution, free of glucose, and the respirometers were charged as follows:

Control..... ½ cc. stock yeast suspension plus
2½ cc. normal Ringer solution

Experimental.. ½ cc. stock yeast suspension plus
2½ cc. Ringer solution containing irradiated glucose

After a period of 40 min. the experimental showed an increase in respiration of 16 per cent over that of the control. This figure, being considerably in excess of experimental error, indicates that if the glucose in Ringer solution is irradiated, a greater oxygen uptake results than if the glucose is not irradiated.

Although this experiment shows that irradiated glucose can stimulate the respiration of yeast, the stimulation is small when compared with that produced by the fraction resulting from the irradiation of a yeast suspension in Ringer solution.

Effect of Irradiating Yeast Cells in Distilled Water

The purpose of this experiment was to determine whether or not it was necessary to suspend the yeast cells in Ringer solution during irradiation.

A stock suspension of yeast was prepared by adding one-fourth cake of yeast (Saccharomyces cerevisiae) to 200 cc. of

distilled water and shaking thoroughly. The suspension was then irradiated for 20 min., centrifuged for 15 min. and the fluid drawn off for use.

The respirometers were charged as follows:

Control . . . . . ½ cc. stock yeast suspension plus
2½ cc. Ringer solution

Experimental . . ½ cc. stock yeast suspension plus
1 cc. Ringer solution plus
1½ cc. irradiated fluid

The respiration was measured over a period of 40 min. and the oxygen consumption of the experimental found to be 17 per cent greater than that of the control.

Although the increase in oxygen consumption was considerably less than in the experiments in which the yeast was suspended in Ringer solution, it was still considerably greater than experimental error. These results, coupled with the results of the previous experiments, show that all the activation does not result from the action of ultra-violet light on the Ringer solution, nor does it all result from the action of ultra-violet light on the yeast cells.

The Relative Stimulative Effect of the Irradiated Centrifuged Fraction and the Non-Irradiated Centrifuged Fraction

The purpose of this experiment was to determine the difference in the stimulative effect between the centrifuged fraction of an irradiated suspension and the centrifuged fraction of a non-irradiated suspension.

About 7 cc. of a stock yeast suspension were irradiated for 20 min. and then centrifuged for 15 min. Another 7 cc. of a stock yeast suspension were centrifuged only. The two preparations were then introduced into the respirometer flasks as follows:

Control	½ cc. stock yeast suspension plus 2½ cc. Ringer solution
Experimental *Irradiated*	½ cc. stock yeast suspension plus 1½ cc. fluid from irradiated suspension plus 1 cc. Ringer solution
Experimental *Non-irradiated*	½ cc. stock yeast suspension plus 1½ cc. fluid from non-irradiated suspension plus 1 cc. Ringer solution

The respiration was measured over a period of 30 min. The irradiated fraction gave an increase of 78 per cent oxygen uptake over that of the control, while the non-irradiated fraction gave a 43 per cent increase over that of the control.

From these data it is clear that if yeast is suspended in Ringer solution, a fraction is produced which stimulates the respiration of a yeast suspension to which it is added; however, irradiated yeast produces this fraction to a greater degree than non-irradiated.

It cannot be logically deduced that a stimulative fraction was liberated by the living yeast cells as might be concluded from the results obtained with the centrifuged non-irradiated suspension, for there must naturally have been present a certain percentage of dead yeast cells. It is quite probable that the stimulative fraction was liberated from the dead cells, although further tests are necessary to establish the origin of the fraction.

SUMMARY

1. Both the centrifuged and filtered fluids from irradiated yeast suspensions were tested for their power to stimulate the respiration of non-irradiated yeast. The stimulation of the cell-free fluid was found to be pronounced while that of the washed cells was negligible.

2. The influence of prolonged irradiation on the stimulative factor was studied. It was found that the stimulative factor produced by the irradiation of yeast could neither be destroyed, nor increased above a given value, by prolonged irradiation.

3. Studies were conducted to determine if the stimulative factor would deteriorate on standing. It was proved that the stimulative factor is extremely stable with regard to time, showing no deterioration over a period of 117 hr.

4. Investigations which were conducted in this research prove that the stimulative factor itself did not consume oxygen.

5. The stimulative factor obtained through the irradiation of a fresh yeast suspension was compared with that obtained through the irradiation of an old yeast suspension for their power to stimulate the respiration of a freshly prepared suspension. The fraction prepared from fresh yeast was found to be more potent than that prepared from the old yeast; however, the difference could not be considered significant.

6. Irradiated glucose in the Ringer solution was tested for its power to stimulate the oxygen consumption of yeast. Although found to have definite stimulative power, the irradiated glucose did not possess it to the same degree as did the fluid obtained from irradiated yeast suspensions.

7. Studies were conducted to determine if the centrifuged fluid from the *non-irradiated* yeast suspensions contained a stimulative factor. The results showed that *non-irradiated* suspensions contain the stimulative factor, but to a considerably less degree than the *irradiated* suspensions.

CONCLUSIONS

The respiratory stimulating factor produced by the action of ultra-violet light on yeast cells can be separated from the cells either by centrifuging or by passage through a Seitz filter. This factor is extremely stable, showing no deterioration

when allowed to stand for prolonged intervals of time. A limited amount of this respiratory stimulating factor is present even in a suspension of yeast cells which has not been irradiated.

Ringer solution or, more specifically, the glucose in Ringer solution, when subjected to ultra-violet light, has the power to stimulate cell respiration. It possesses this power, however, to a much less degree than the cell-free fraction from an irradiated suspension of cells in Ringer solution.

A STUDY OF STIMULATION OF GROWTH, RESPIRATION AND FERMENTATION BY BIOS AND BIOS-LIKE SUBSTANCES

By ROBERT J. NORRIS and SISTER M. VERONITA RUDDY, O.P.

The literature is replete with references to the stimulation of *growth* and *fermentation* of yeast suspensions by bios,[1] while but little has been written concerning the influence of bios on *respiration.*

A search of the literature disclosed, however, that Copping[2] had tested the effect of bios upon respiration of yeast cells and obtained negative results by the direct addition of bios to yeast suspensions.

It has been demonstrated repeatedly in this laboratory that when yeast suspensions in Ringer phosphate solution are irradiated, substances are produced in the solution which are capable of stimulating respiration, growth and fermentation of either the cells surviving the treatment or the untreated cells to which the irradiated cell-free fluid may be added.

In order to demonstrate the activation of growth, respiration and fermentation of yeast cells by ultra-violet irradiation, the following conditions must be fulfilled:

1. The irradiation time must be sufficient to kill enough of the yeast cells to produce an adequate supply of the stimulating substance.

2. The surviving yeast cells must be in such number that when stimulated by the substances produced from the dead

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[1]Fulmer, E. I., *Iowa Acad. Sci.*, **31** (1924); Wallace, G. I., and Tanner, F. W., *Zentr. Bakt. Parasitenk.*, **76** (1928): 1; Wynd, F. L., and Reynolds, E. S., *Ann. Missouri Botan. Garden*, **22** (1935): 771.

[2]Copping, A. M., *Biochem. J.*, **23** (1929): 1050.

cells, the growth, fermentation and respiration become greater than those of a control sample.

The fulfillment of these conditions necessitates a careful adjustment of the time of irradiation of the suspension as well as the use of a proper substrate. It is therefore not surprising that investigators, by adopting various arbitrary conditions of irradiation, should have failed to make this observation. Such accurate control is not essential if all determinations (growth, fermentation and respiration) are based upon the number of surviving cells rather than the total number of cells, for, as was stated in an earlier paper,[3] the respiration of the total irradiated cell suspension may be less than the control, while the respiration per surviving cell may be found to be increased. These precautions are largely unnecessary if the second method is employed, i.e., when the cell-free liquid of the irradiated cells is tested upon untreated cells.

The present paper attempts to determine if respiration can be stimulated by bios and, if so, whether this stimulation is similar to that produced by the cell-free fraction of irradiated yeast suspensions. Another object is to study the comparative stimulating effects of bios and the cell-free fraction of irradiated yeast on growth and fermentation of yeast suspensions as a means of determining if bios and the stimulative fraction resulting from the irradiation of yeast suspensions are identical.

It is a further object of this research to determine if the stimulation of growth, respiration and fermentation by bios-like substances is the result of a single factor or of several factors. Is one factor responsible for the stimulation of respiration, another for the stimulation of growth, and still another for fermentation?

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[3]Fardon, J. C., Carroll, Sr. M. Jordan, O.P., and Ruddy, Sr. M. Veronita, O.P., p. 17 this issue.

EXPERIMENTAL

Stimulation of Respiration by Bios

Although Copping[4] detected no *immediate* increase in respiration upon the addition of bios to a suspension of yeast cells, she did detect an increase after 24 hr. of incubation. She was, however, unable to state whether the increased respiration was the result of the direct action of bios or of the multiplication of the cells.

Bios fractions were prepared by the method of Narayanan[5] and tested for their power to stimulate the respiration of suspensions of yeast cells.* The addition of this crude preparation to untreated yeast cells resulted in a marked stimulation of respiration of the cells, confirming the belief that bios bears a marked similarity to the fraction produced by the irradiation of suspensions of yeast cells.

The results of our experiments make it difficult to explain why the material used by Copping failed to produce stimulation of respiration. Our stimulation of respiration, by both the bios prepared by the method of Narayanan and by our irradiated cell-free fraction, was so clearly marked that it seemed hardly possible Copping could have failed to observe it. Since our experiments failed to verify those of Copping, it was believed that both our process of irradiation and the method of Narayanan must have produced a factor which was different from hers. In view of the contradictory results, it was decided to repeat the experiments of Copping, following her procedure as closely as possible.

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[4]Copping, A. M., *loc. cit.*

[5]Narayanan, B. F., *Biochem. J.*, 24 (1930): 16.

*This work has been repeated, using an entirely different method for fractionation of bios to determine if the results presented herein depend in some degree upon the manner of fractionation of the active material. This paper will appear shortly.

A commercial sample of marmite such as used by Copping[6] was procured. The marmite preparation was tested at the same concentration as that used in our previous experiments, i.e., 3.3 mg./cc. of yeast suspension. A stimulation of respiration amounting to 90 per cent was obtained. These seemingly contradictory results will be discussed later in this paper.

Preparation of Irradiated Cell-Free Fluid

One hundred grams of Fleischmann baker's yeast (Saccharomyces cerevisiae) were suspended in 1000 cc. of Ringer phosphate glucose solution and shaken well. The mixture stood for 3 days and was shaken at frequent intervals. At the end of this time it was irradiated for a period of 20 min., at a distance of 12 in., under an unfiltered quartz mercury arc, in layers 1 cm. deep, with constant rocking of the solution. The lamp was operated at 75 volts across the terminals. The optimum time and conditions of irradiation for the production of the stimulating fraction have not as yet been established. However, the technic described above resulted in the production of a very active extract. The mixture was centrifuged, filtered through paper and then equal portions filtered through Seitz and Berkefeld filters, and preserved in the ice chest under sterile conditions. No loss of potency was observed over a period of several months. This preparation will be referred to as "irradiated yeast fluid." The fraction was usually a water-clear fluid, sometimes faintly yellow in color, possessing a definitely acrid odor.

From Table I it is clear that in our experiments marmite, as employed by Copping, as well as bios prepared by the method of Narayanan, produced marked stimulation of respiration. It is also apparent that the stimulation of respiration is not a direct function of the concentration of the stimulating fraction.

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[6]Copping, A. M., *loc. cit.*

TABLE I

Effect of Marmite, Crude Bios and Irradiated Yeast Fluid on Stimulation of Respiration

Source of Bios	Mg. Per cc. of Yeast Suspension					
	1.56	3.125	3.3	6.25	12.5	25
	Per Cent Increase in O_2 Uptake					
Marmite	...	...	90	...	...	...
Crude Bios (Narayanan)	78	102	...	122	139	176
Irradiated Yeast Fluid	81	62	...	113	175	...

Referring to Table I, it will be noted that with the addition of 1.56 mg. of the active factors the crude bios shows a greater potency than the irradiated yeast fluid. However, with the addition of 12.5 mg. of the active factors the order is reversed, showing the irradiated yeast fluid to be the more potent. These results would seem to indicate that crude bios, produced by the method of Narayanan, may not be the same substance as the respiratory factor produced from irradiated yeast.

Influence of Sugar on the Stimulation of Yeast Respiration by Bios

During the preparation of bios from cane sugar according to the method of Funk and Freedman,[7] an observation was made which undoubtedly explains the failure of Copping to observe stimulation of respiration by bios. Our preparation of bios from cane sugar failed to give any stimulation of respiration when tested by our routine methods. Since this bios preparation, which was very crude, contained considerable cane sugar, it occurred to us to try the effect of cane sugar alone upon another bios preparation (marmite) which we had previously found capable of increasing the respiration of yeast. The addition of cane sugar to a marmite-yeast suspension im-

[7]Funk, C., and Freedman, L., *J. Biol. Chem.*, 56 (1934): 851.

mediately obliterated any stimulation of respiration. A control suspension plus cane sugar gave just as intense a respiration as a yeast suspension containing marmite in addition to the cane sugar. This emphasizes the importance of fasting the yeast cells when demonstrating the stimulation of respiration by bios-like substances.

The mechanism of this stimulation of respiration of the fasting yeast cell is of considerable interest. Our observations on this point will be reported later.

Effect of Heat on Irradiated Cell-Free Fluid

It is well known that the bios complex is quite stable to heat, alkalis and oxidizing agents.[8] With this in mind, it was decided to determine the effect of heat upon the stability of our irradiated fluid with the special object of ascertaining the effect of heat upon the power of the fraction to stimulate respiration. The results are shown in Table II.

TABLE II

Effect of Heat upon Stability of Respiratory Stimulating Factor of Irradiated Yeast Fluid

Sample	O_2 Uptake In cu. mm.	Per Cent Stimulation
Control (Ringer solution plus yeast suspension)	59.53	.....
Autoclaved irradiated extract plus yeast suspension	176.42	196.3
Irradiated extract plus yeast suspension	177.98	198.9

The results of this experiment indicate no change in activity after autoclaving at 20 lb. pressure for 20 min.

[8]Euler, Hans v., and Larsson, H., *Z. physiol. Chem.*, 223 (1934): 189

Effect of Dialysis

As bios is known to be readily dialyzable and as von Euler[9] had shown that his factor Z and bios II apparently dialyze at the same velocity, we were interested in determining whether the substance, or substances, in irradiated yeast fluid manifested the same characteristics.

Fifty cc. of a very potent irradiated yeast extract were placed in a collodion bag, prepared in the usual manner, and the fraction was dialyzed for 24 hr. in running distilled water at room temperature. At the end of this time the volume had increased to 80 cc. The material was removed from the bag and 9 cc. of 10x strength Ringer solution plus 4.5 cc. of buffer were added to restore the inorganic salts lost by the dialysis. The pH was then 7.3. A 50 cc. control portion not dialyzed was diluted to the same volume with Ringer solution. One cc. of each was tested upon yeast suspension. The results are shown in Table III.

TABLE III

Effect of Dialysis upon Stimulation of Respiration of Irradiated Yeast Fluid

Sample	O_2 Uptake In cu. mm.
Control (Yeast suspension plus Ringer)	65.13
Dialyzed Irradiated Extract (Material remaining after dialysis) plus Yeast Suspension	75.40
Non-Dialyzed Irradiated Extract plus Yeast Suspension	214.95

This experiment demonstrates that the factor in irradiated yeast extract responsible for the stimulation of yeast respiration passes through the dialyzing membrane, and in this property resembles bios.

[9]Euler, Hans v., and Larsson, H. Z., *loc. cit.*

Effect of Irradiated Yeast Fluid upon Growth and Fermentation of Yeast

Growth—Our observations upon the effect of irradiated yeast fluid in promoting growth of yeast in Reader's media are largely qualitative. No accurate assay for the exact potency of growth-promoting power was made. In dosages of approximately 1 mg. per cc. of inoculated media the count changed from 1 to 50 in a period of 48 hr., whereas there was little if any change in count in similar media to which the extract had not been added.

Fermentation—Our early experiments upon fermentation were carried out by adding the irradiated extract to a suspension of yeast in Laurent's media contained in Durham fermentation tubes. There was a marked increase in the rate of fermentation. These experiments were then repeated, using Warburg apparatus (respirometers) for measuring the rate of fermentation. In these experiments the rate was accelerated approximately 95-100 per cent by the addition of 1 mg. (1.5 cc. extract) of the active material.

Are Separate Substances Responsible for the Stimulation of Growth, Respiration and Fermentation?

These experiments were carried out with the object of determining whether the stimulation of respiration, growth and fermentation was the result of the activity of a single or of multiple substances. If these effects are the result of a single substance, then, obviously, when different samples are tested for their power to stimulate growth, respiration and fermentation the ratios for the three properties should be roughly the same for all samples tested. If, however, we are dealing with multiple substances, the order of activity might be subject to a decided variation. At the time of these experiments our technic had not been standardized to a sufficient

degree to make possible a numerical evaluation of potencies. Hence, we can only present the data on growth, respiration and fermentation of the irradiated extract and the bios fractions in the order of potency found.

As stated elsewhere, the bios was prepared according to the method of Narayanan. Samples of the fractions were removed for assay at each step in the process of concentration. Considerable difficulty was experienced in securing a sharp and complete precipitation of bios with the phosphotungstic acid reagent. It was necessary to carry out this precipitation twice in order to remove all precipitable matter. The fractions were numbered according to the steps at which they were removed and are designated as follows:

Sample No.	1.	First alcoholic extraction
" "	2.	Combination of first and second alcoholic extractions
" "	3.	After baryta precipitation
" "	4.	After lead acetate precipitation
" "	5A.	Material from first phosphotungstic acid precipitation
" "	6A.	Material from second phosphotungstic acid precipitation
" "	5B.	Material adsorbed to $BaSO_4$ precipitate after decomposition of first phosphotungstic acid precipitation
"	I. Y. Ext.	Irradiated yeast extract prepared by standard technic

Table IV shows the activity of these fractions when tested simultaneously for their action on growth, respiration and fermentation.

TABLE IV

Comparison of Stimulation of Growth, Fermentation and Respiration by Irradiated Yeast Extract and Bios Fractions

Order of Potency	Growth	Respiration	Fermentation
1 (maximum)	Sample 2	Sample 6A	Sample 1
2	" 3	" 4	" 2
3	" 4	" 1	" 3
4	" 1	" I. Y. Ext.	" 4
5	" I. Y. Ext.	" 2	" 6A
6	" 6A	" 5A	" 5A
7	" 5A	" 3	" I. Y. Ext.
8 (minimum)	" 5B	" 5B	" 5B

If bios is a single substance capable of promoting growth, respiration and fermentation, it would be expected that the fraction showing highest potency in the stimulation of growth would also show the highest potency in the stimulation of respiration and fermentation, assuming that no specific inhibiting factors were present. From an examination of Table IV it will be seen that Sample No. 2 has the maximum growth-promoting power, while Sample No. 6A has the maximum power of stimulating respiration, and Sample No. 1 the maximum power for the stimulation of fermentation. Sample No. 5B, showing minimum stimulative power, is the only one which shows the same order of potency when tested for growth, respiration and fermentation.

These results seem to indicate that in a crude preparation of bios one substance alone is not responsible for the stimulation of growth, respiration and fermentation.

SUMMARY

1. Bios, prepared by the method of Narayanan and marmite as employed by Copping, stimulate the respiration of yeast (Saccharomyces cerevisiae) when added to suspensions of this

yeast. In this respect, bios is similar to the cell-free fraction of irradiated yeast.

2. An excess of sugar in the media in which the yeast is suspended can completely obliterate the respiratory stimulating power of bios-like substances.

3. The respiratory stimulating factor produced by the action of ultra-violet light on yeast cells is stable to heat and in this respect resembles bios.

4. The respiratory stimulating factor produced by the action of ultra-violet light on yeast cells is similar to bios in that it is readily dialyzable.

5. The dialyzable fraction from irradiated yeast has the power to stimulate growth and fermentation as well as respiration.

6. Evidence has been cited to indicate that the growth, fermentation and respiration stimulating factors are not identical.

CONCLUSIONS

Contrary to the observations of some early workers in this field, bios has the power to stimulate the respiration of yeast. This stimulative power, however, can be readily obliterated by the presence of an excess of sugar.

The growth, fermentation and respiration stimulating factors produced by the irradiation of yeast are in many respects similar to bios. However, from the results cited herein it is not safe to conclude that the substances are identical.

Our results seem to indicate that three separate factors are responsible for the stimulation of respiration, growth and fermentation.

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BIOLOGICAL ASSAY OF DIALYZED FRACTIONS OF BIOS

By R. J. NORRIS and SISTER MARY JANE HART, O.P.

Among those substances which affect the functional activities of cells is "Bios," designated as such by Wildiers[1] at the time of its discovery by him. He claimed it to be an organic substance (or substances) essential for the proliferation of yeast cells and mentions the properties by which it can be easily recognized. The property which is of particular interest to us is its dialyzability.

Pasteur,[2] who was among the earliest investigators in this field, although not recognizing bios as such, had demonstrated that yeast cells stimulated the growth rate as well as the fermenting power of other yeast cells. In these early experiments the question naturally must have arisen whether one or multiple substances were responsible for growth and fermentation.

In 1922 Fulmer and Nelson[3] claimed to have separated bios into bios A and bios B, each of which stimulated growth to a certain degree, but proved more potent when in combination. About the same time Ide[4] showed bios to be a fermentation factor and doubted its relation to growth activity; in 1924, Miller[5] showed that it could be separated into bios I and bios

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[1]Wildiers, E., *La Cellule*, 18 (1901): 313.

[2]Pasteur, L., *Ann. chim. phys.*, 3rd Series, 58 (1860): 323; 4th Series, 25 (1872): 145; *Compt. rend.*, 73 (1871): 1419; 78 (1874): 213.

[3]Fulmer, E. I., and Nelson, V. E., *Proc. Iowa Acad. Sci.*, 29 (1922): 371.

[4]Ide, M., *Zentr. Bakt. Parasitenk.* II, 18 (1907): 193; *J. Biol. Chem.*, 46 (1921): 521.

[5]Miller, W. L., *Science*, 59 (1924): 197.

II, bios I being inactive without the presence of bios II, but supplementing the activity of bios II in the presence of the latter. More recently Eastcott, working with Miller, identified bios I as inositol. As late as 1932 Miller[6] separated bios II into fractions A and B. Kogls designated factor Z as the fermentation stimulator and von Euler[7] later claimed this to be identical with bios II. Von Euler found that the acceleration of fermentation parallels the acceleration of growth, and even after dialysis of the solutions through collodion membranes, the proportion of this increase remains the same. Despite the similarity in the rates of dialysis, von Euler does not conclude that the growth and fermentation factors are identical.

Williams and Truesdail,[8] in 1931, by the use of collodion bags, dried in 40, 60 and 80 per cent alcohol, showed the acid factor (accumulated at the anode) to be readily dialyzable through all the membranes, while the basic factor dialyzed but slowly through the bag treated with 40 per cent alcohol, and very little through the others. These observers claim that the material which passed through the membranes probably contained portions of both factors and showed growth-stimulating activity. The undialyzable material, however, had practically no effect on yeast growth.

Little work has been done on bios to prove its power to stimulate the *respiration* of cells[9] In a preceding paper in this publication[10] experiments have been reported which show that bios and bios-like substances have the property of stimulating respiration. These earlier researches deal with growth and

[6]Miller, W. L., Eastcott, E. V., and Sparling, E. M., *Trans. Roy. Soc. Canada*, Sec. III (1932): 165.

[7]Euler, H. von, and Larsson, H., *Zeit. physiol. Chem.*, 114 (1922): 4.

[8]Williams, R., and Truesdail, J. H., *J. Am. Chem. Soc.*, 53 (1931): 4171.

[9]Copping, A. M., *Biochem. J.*, 23 (1929): 1050.

[10]Norris, R. J., and Ruddy, Sr. M. Veronita, O.P. This issue, p. 53.

fermentation as well as respiration and seem to indicate the existence of various fractions separately responsible for the three properties. In view of these earlier results an attempt was made to separate these hypothetical active factors by means of dialysis.

In our work a concentrated yeast extract was employed and dialyzed to study whether or not the factors responsible for fermentation, respiration and growth diffuse at the same rate, and thus show a possible relation between the fractions.

EXPERIMENTAL

Preparation of Fractions—In the preparation of the crude bios the first step of Narayanan's[11] method was used. This consisted in extracting 7 lbs. of yeast with 2.5 liters of 95 per cent alcohol for 4 hrs. at 60° to 70° C., during which time the mixture was constantly agitated. The mixture was then centrifuged for 8 min. and the liquid decanted and filtered. The filtrate was concentrated by vacuum distillation to remove all alcohol. During the process of concentration some difficulty was encountered in preventing the material from foaming over. This was avoided by careful control of the pressure.

After sterilization of this yeast extract, approximately 150 cc. were transferred by means of sterile pipettes into a collodion bag previously prepared after the manner outlined by Morrow.[12] The bag was so arranged that it could be easily suspended in a beaker containing glass-distilled water. Within the beaker a glass stirrer was kept rotating throughout the entire period (64 hrs.). At regular intervals (after ½ hr., 1 hr., 2 hrs., 4 hrs., etc. to 64 hrs.), samples were taken with sterile apparatus from within the bag and from the dialysates. When all samples had been taken they were resterilized and it was

[11]Narayanan, B., *Biochem. J.*, 24 (1930): 6.

[12]Morrow, C. A., Biochemical Laboratory Methods (1935).

noted that in all the samples dialyzed longer than 4 hrs. a precipitate was formed, giving a turbid appearance to the solution.

Attempts were made to dilute to the same potency the samples which were removed after various periods of dialysis. The diluted samples were tested for their powers to stimulate fermentation, respiration and growth of yeast suspensions. It was believed that the active fraction, and only the active fraction, had an absorption band in the visible portion of the spectrum and, therefore, if all samples were diluted to the same color they might show equal potencies.

Fermentation—While making a study of the technic required to give the most accurate results on the stimulation of fermentation by our fractions, it was found that the proper yeast count to be used was of much importance. It was determined by experiment that a count of 80 gave the most accurate and consistent results.

The flasks and tubes of the Warburg manometers and also the materials added to the flasks were flushed with CO_2 in the usual manner, after which they were allowed to run for a 1-hour equilibrium period and a 5-minute fermentation period. It was found that the CO_2 output from the yeast control remained constant for 1 hr., after which it began to fall. Preliminary tests showed that when bios was added there was a lag period in which the CO_2 output was at first retarded, then increased to a maximum at 1.5 hrs., after which time the rate of fermentation fell off sharply. Fermentation was greater than the graduations on our manometers would accommodate if a period longer than 5 min. was used. Other preliminary experiments with more dilute solutions indicated that periods longer than 5 min. did not give constant and reproducible results.

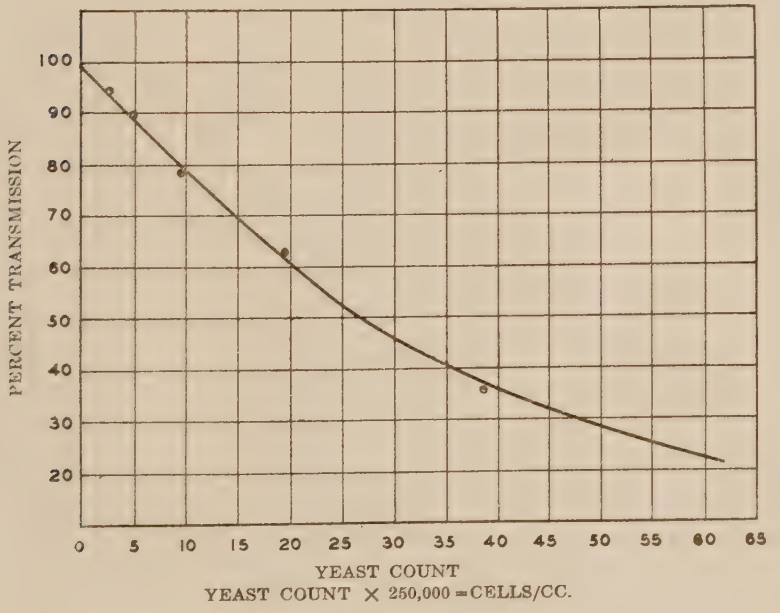
A Ringer phosphate solution containing 12 per cent glucose was added to each manometer flask, bringing the glucose in

the flask to 4 per cent, as required for optimum stimulation of fermentation. In all experiments 1 cc. of the sample to be tested was used. Each flask in the fermentation experiments contained a total of 3.3 cc. of material.

Respiration—The Warburg technic was followed in the respiration experiments. Each flask was charged with 3.3 cc. of material, consisting of 1 cc. of the sample to be tested, 1 cc. of yeast suspension in Ringer solution, 1.1 cc. Ringer phosphate glucose solution, and 0.2 cc. KOH in the inner well. A yeast count of 250, an equilibrium period of 25 min. and a respiration period of 15 min. were used. It had been found earlier that these changes gave the most accurate and consistent results.

Growth—The yeast counts upon which the dialysates were tested were determined by means of a photodensitometer,

FIG. 1—*Yeast Count as a Function of Light Transmitted*



designed by Beck[13] of this laboratory. A calibration curve, in which the number of cells is plotted as a function of light transmission, is reproduced in Fig. 1.

The growth determinations were made according to Fraser's[14] technic, slightly modified. L-shaped rocker tubes were used, making approximately 70 oscillations per minute in the plane of the L through a 30° angle. The water in the bath was kept at a constant temperature of 30° C. Each tube, with exception of the control, was charged with 1 cc. of the sample to be tested. All tubes received 0.5 cc. of a 50 per cent dextrose solution, enough yeast suspension to make the initial count of the solution 1, and Reader's media to bring the total volume in each tube to 25 cc. Photodensitometer readings were taken after 24 hrs.

DISCUSSION

The results in Table I show that there was a gradual increase in potency of the fermentation factor of all the samples dialyzed for 4 hr., at which point maximum stimulation was reached, while the 8-hour sample showed a decided drop in potency.

The results on respiration (Table II) were similar to those of the fermentation experiments. There was a rapid increase in potency with increased time of dialysis up to the 4-hour sample, after which the potency showed a decided decrease.

The results of growth are given in Table III. Like those of respiration and fermentation, they show a maximum potency for the 4-hour sample. There was a minimum at the 8-hour period and thereafter a gradual increase.

A glance at the data in the Tables indicates that, contrary to our expectations, color cannot be used as an index of potencies of our samples. Other experiments, which as yet have not been published, prove that the stimulative actions of

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[13]Beck, W. A., *Science*, 85 (1937): 2206.

[14]Fraser, C. G., *J. Phys. Chem.*, 25 (1921): 4.

TABLE I

Stimulation of Fermentation by Dialyzed Fractions

Sample	Hours Dialyzed	Dilutions	Mg. of Solid Content per cc. Tested	F_c (Cu. mm. CO_2 Production of Control)	F_e (Cu. mm. CO_2 Production of Experimental)	$F = \frac{F_e - F_c}{F_c} \times D$	$\frac{F}{\text{Mg. of Solid Content per cc.}}$
No. 1	½	1–10	0.92	78.46	147.412	10.20	11.10
" 2	1	1–80	0.316	"	112.285	34.48	108.96
" 3	2	1–160	0.371	"	143.418	132.32	359.80
" 4	4	1–320	0.260	"	155.68	315.20	1212.80
" 5	8	1–320	0.295	"	103.96	105.32	353.28
" 6	16	1–320	0.285	"	102.308	97.28	341.76
" 7	32	1–320	0.260	"	100.094	88.32	339.20
" 8	64	1–320	0.318	"	98.861	83.200	261.44

TABLE II

Stimulation of Respiration by Dialyzed Fractions

Sample	Hours Dialyzed	Dilutions	Mg. of Solid Content per cc. Tested	R_c (Cu. mm. O_2 Uptake of Control)	R_e (Cu. mm. O_2 Uptake of Experimental)	$R = \frac{R_e - R_c}{R_c} \times D$	$\frac{R}{\text{Mg. of Solid Content per cc.}}$
No. 1	½	1–10	0.92	42.05	77.626	8.45	91.90
" 2	1	1–80	0.316	"	67.958	49.20	155.60
" 3	2	1–160	0.371	"	60.616	68.80	185.60
" 4	4	1–320	0.260	"	61.309	145.92	561.60
" 5	8	1–320	0.295	"	54.549	95.04	322.560
" 6	16	1–320	0.285	"	54.926	97.92	343.04
" 7	32	1–320	0.260	"	52.179	77.12	296.80
" 8	64	1–320	0.318	"	54.859	97.28	305.60

TABLE III

Stimulation of Growth by Dialyzed Fractions

Sample	Hours Dialyzed	Dilutions	Mg. of Solid Content Per cc. Tested	G_c (Yeast Cells per cc. Control)	G_e (Yeast Cells per cc. Experimental)	$G = \frac{G_e - G_c}{G_c} \times D$	$\frac{G}{\text{Mg. of Solid Content per cc.}}$
No. 1	½	1–10	0.92	8.9	26.84	20.18	21.90
" 2	1	1–80	0.316	"	17.279	75.28	238.40
" 3	2	1–160	0.371	"	15.698	122.24	329.60
" 4	4	1–320	0.260	"	17.599	313.80	1203.20
" 5	8	1–320	0.295	"	15.239	227.84	771.20
" 6	16	1–320	0.285	"	15.555	240.00	853.20
" 7	32	1–320	0.260	"	15.89	241.52	966.40
" 8	64	1–320	0.318	"	17.46	307.84	967.36

our fractions, whether they be tested on growth, respiration or fermentation, are not directly proportional to the concentration of the fractions. This lack of direct relationship between the concentration and the stimulating powers rendered it impossible to make *absolute* comparisons between the potencies of our samples. However, since our problem was one of determining whether or not the growth, respiration and fermentation factors dialyzed at the same rate, we were interested only in *relative* values. In the curves in Figs. 2 and 3 are plotted what might be termed indices of growth (G), fermentation (F), and respiration (R). These indices have been determined in the following manner:

For curves in Fig. 2:

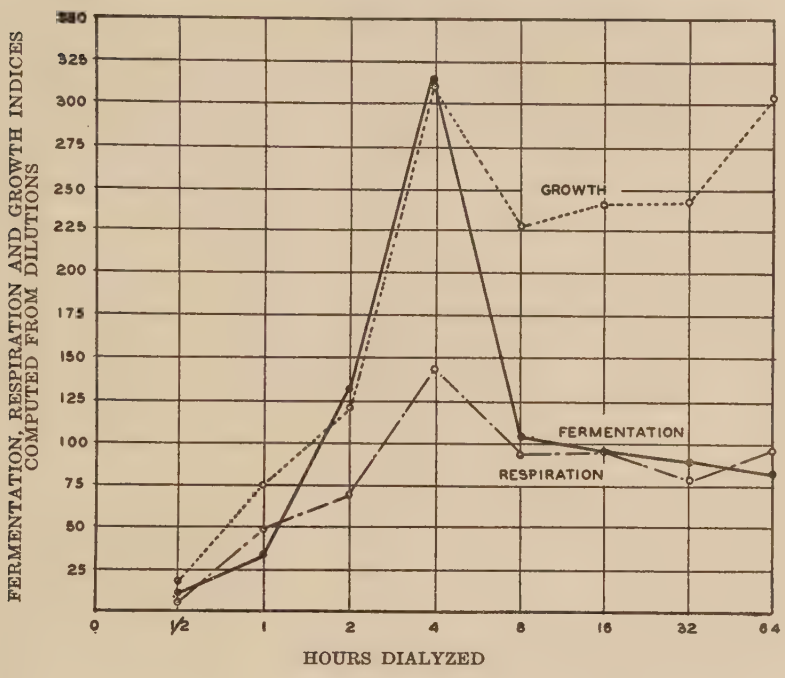
$$G = \frac{G_e - G_c}{G_c} \times D$$

$$F = \frac{F_e - F_c}{F_c} \times D$$

$$R = \frac{R_e - R_c}{R_c} \times D$$

G_e = Growth of experimental
G_c = Growth of control
F_e = CO_2 production of experimental
F_c = CO_2 production of control
R_e = O_2 uptake of experimental
R_c = O_2 uptake of control
D = Number of times sample was diluted before testing

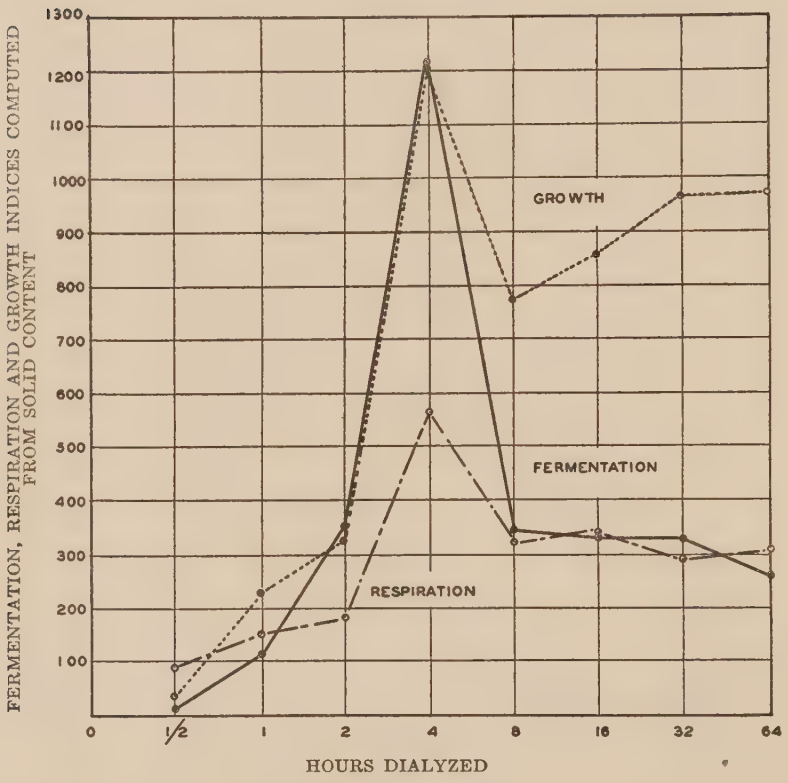
FIG. 2—*Comparative Stimulation of Fermentation, Respiration and Growth by Dialyzed Fractions Based on Dilutions*



The parallel dialysis of the three factors from the ½-hour to the 8-hour samples, Figs. 2 and 3, each resulting in a maximum at the end of the 4-hour interval, indicates the similarity of the fermentation, respiration and growth factors. In so far as growth and fermentation are concerned, our results confirm those of Williams and Truesdail, and von Euler. From the position of this maximum, it seems that either a single factor is responsible for the three activities or that the stimulative material for each function dialyzes at the same rate, thus showing similarity in physical properties.

Samples of the dialysate taken from 8 hrs. to 64 hrs. might indicate slightly different properties for the three factors. The 64-hour sample shows a slight decrease in fermentation and

FIG. 3—*Comparative Stimulation of Fermentation, Respiration and Growth by Dialyzed Fractions Based on Weight of Solid Content*



For curves in Fig. 3:

G, F and **R** have been divided by the solid content per cc. of the samples tested in order to give an index more nearly proportional to the dry weight of the dialysates.

respiration as compared with the 16-hour sample, while growth shows an increase. Some of these variations, however, are in all probability within the limits of experimental error, and leave this portion of the curve open for further investigation. A general consideration of the results of these experiments shows

only that the active factors are dialyzable, but does not determine the number of factors responsible for stimulation.

It may be that the precipitates in the samples dialyzed for the longer periods carried with them some of the potent material. This may account for the drop in potency and the other variations in the samples dialyzed longer than 8 hrs. The precipitates which developed during the 8-hour to the 64-hour dialysis may be responsible for the differences in the slopes of the curves during this interval. If the accuracy of the curves can be relied upon, the differences in slopes might indicate that the factor responsible for growth is different from the factor responsible for fermentation and respiration.

No work has been done to test the relative values of the precipitate and supernatant liquid.

CONCLUSIONS

These experiments confirm the claim of Williams and Truesdail that the active factor or factors responsible for *growth* and *fermentation* are readily dialyzable. They support the conclusions of von Euler that the fermentation and growth factors possess closely related properties.

The factor responsible for *respiration,* like the factors responsible for growth and fermentation, is readily dialyzable.

Although the curves reach maxima and minima at the same time, the slopes of the curves are so widely divergent that there seems to be no justification for the conclusion that the fermentation, respiration and growth factors are one and the same substance.

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NOTE ON THE ULTRA-VIOLET ABSORPTION SPECTRA OF BIOS AND OTHER GROWTH PROMOTING PREPARATIONS

By JOHN R. LOOFBOUROW, REV. LAWRENCE SCHMIEDER, SISTER MIRIAM MICHAEL STIMSON, O.P., and SISTER CECELIA MARIE DWYER, S.C.

Fardon, Norris et al.[1] have demonstrated in this laboratory that various kinds of cells killed by ultra-violet, X-rays and other methods produce factors which stimulate respiration, fermentation and growth. Preparations having similar properties prepared from normal yeast and other sources have been known for some time and have been given the name "Bios." It was considered of interest to study the ultra-violet absorption spectra of bios preparations from various sources and of the stimulating fractions from killed cells, prepared in various ways, in order to investigate whether one or more active materials were present in these preparations and to help in the elucidation of the chemical structure of the active constituent or constituents.

EXPERIMENTAL

In this preliminary study, four preparations only were used. One was a cell-free extract from ultra-violet irradiated liver pulp which showed marked stimulation of respiration of yeast. The other three were bios preparations: one made from yeast, and the other two made from malt combings and sucrose respectively. These preparations had been tested for their growth or respiration-promoting activity by those who made them.

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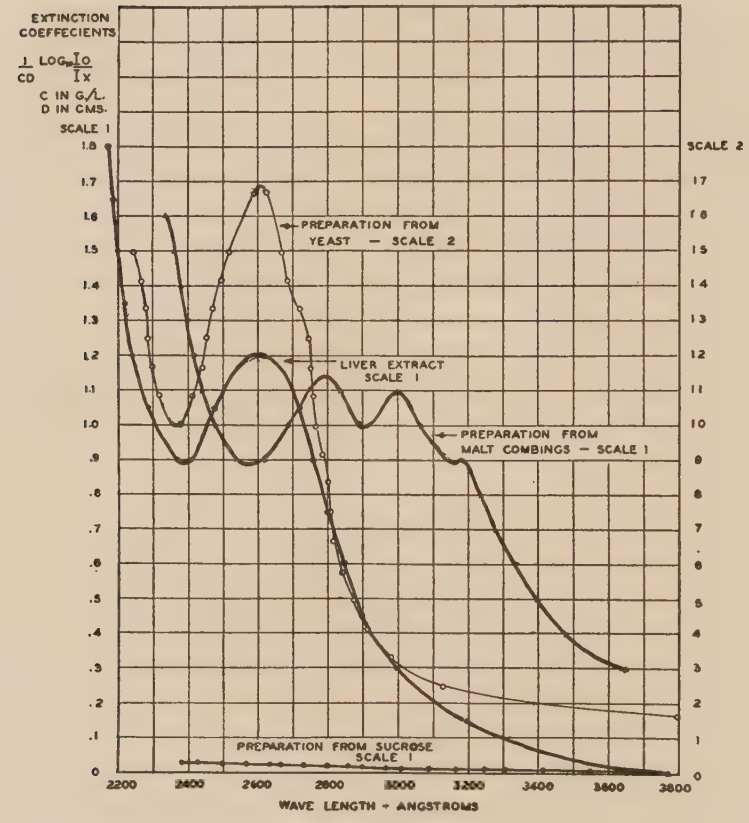
[1]Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sister M. Veronita, O.P., *Nature*, 139 (1937): 589.

The spectra were determined in water solution, using a Hilger quartz spectrograph and Spekker photometer. No accurate determinations of pH were made, but the solutions were all approximately neutral. The spectra are shown in the accompanying figure.

Ultra-Violet Absorption Spectra of Various Bios-Like Preparations



CONCLUSIONS

The spectrum of the bios preparation from yeast resembled that of the liver extract, both having a marked absorption maximum at about 2600 Å, which is characteristic of nucleic acid and its various purines and pyrimidine constituents.[2] The spectrum of the preparation from malt combings lacked any marked extinction at 2600 Å, but showed instead peaks at 2800 and 3000 Å, which may be accounted for in part by the presence of amino acids. The spectrum of the bios preparation from sucrose showed only "end" absorption.

From these data, it is only possible to say that different materials were present in at least three of the preparations. Which of these materials were the active constituents and which were impurities remains to be determined. For that purpose, parallel biologic and spectrographic tests are in progress on preparations made in a variety of ways from different sources. The results of these investigations will be reported later.

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[2]Heyroth, F. H., and Loofbourow, J. R., *J. Am. Chem. Soc.*, 56 (1934): 1728

THE ABSORPTION OF VITAMIN D THROUGH THE SKIN*

By AGNES C. HELMER and REV. CORNELIUS H. JANSEN

The absorption of vitamin D through the skin has been the subject of study for a number of years. Although perhaps not consciously, the first demonstration of the curative value of vitamin D was by means of its absorption through the skin. In the light of present knowledge it is realized that the curing of rickets by sunlight and by artificial ultra-violet light is actually the curing of rickets by the absorption of vitamin D through the skin.

The earliest record of the observation of the curing of rickets by irradiation was made by Palm,[1] who pointed out in 1860 that rickets is prevalent wherever there is little sunlight and unknown or comparatively rare wherever sunshine is abundant. His observation was later verified by Raczynski,[2] who experimented with rickets in puppies and reported in 1912: "It is the sun which plays the principal rôle in the etiology of rickets." He was able to prove that sunlight has a beneficial effect on mineral metabolism and demonstrated this by curing rickets in puppies through exposure to sunlight. Huldschinsky,[3] in 1919, working along the same lines, reported the curing of rickets in children by artificial ultra-violet rays. Chick[4] and her co-workers of the Lister Institute and Medical Research

*This research was conducted under The Andrew Jergens Fellowship, Cincinnati, Ohio.

[1]Harris, L. J., Vitamins in Theory and Practice, Cambridge University Press (1935): 110.

[2]Raczynski, J., *Compt. rend. assocn. intern. pediatrie* (1913): 308.

[3]Huldschinsky, K., *Deut. med. Wochschr.*, 45 (1910): 712.

[4]Chick, H., et al., Med. Research Council (*British*) *Special Rept. Series*, No. 77 (1919-1922): 230.

Council reported similar results in the same year, and in 1920 Hess, in conjunction with Unger,[5] also proved that rickets in children can be cured and prevented by exposure to ultra-violet rays, both artificial and natural.

These researches seemed to indicate that ultra-violet light might function as an equivalent of vitamin D. Hume[6] (1922) states: "Early cessation of normal growth followed by a long period of maintenance might almost be regarded as a defense mechanism, which enabled the rat to tide over a period of vitamin A famine, such as might easily be a danger in the normal life of a rat. When irradiation is applied it would seem as if, either the low level of vitamin A metabolism is rendered sufficient, i.e., the vitamin is economized—or else the vitamin stores are forced to yield up vitamin A at a rate sufficient to produce normal growth until the stores are wholly exhausted. . . . Ultimately, when presumably all the stores are exhausted, no normal growth can be evoked by irradiation. Hence it is clear that light can neither create or be substituted for the vitamin; it appears only to act as an economiser or activator when it is already present."

It is to be remembered that in 1922 vitamin A had not been clearly separated into vitamins A and D, and that growth promotion was generally accepted as a measure of antirachitic activity. Hume's research, indirect as it may seem, is probably the first inference to the absorption of vitamin D through the skin.

In 1923, Goldblatt and Soames[7] reported the following observation: ". . . if rats are kept on a diet deficient in vitamins A and D until they have ceased gaining weight for about four weeks their livers, fed to other rats, do not possess growth-

[5]Hess, A. F., and Unger, L. J., *Proc. Soc. Exptl. Biol. Med.*, 18 (1921): 298; *J. Am. Med. Assoc.*, 77 (1921): 39.

[6]Hume, E. M., *Lancet* II (1922): 1318.

[7]Goldblatt, H., and Soames, K. M., *Biochem. J.*, 17 (1923): 446.

promoting properties; but if, after complete cessation of growth, rats are irradiated for a few weeks by means of a mercury-vapor quartz lamp, their livers acquire the property of stimulating for a short time the gain in weight of rats that have ceased growing on a diet deficient in the fat-soluble vitamin." Powers, Park and Simmonds[8] concluded (1923) from their work that: "Previous experiments have shown that the unknown factor in cod-liver oil which promotes normal formation of bone (and in that sense may be spoken of as antirachitic) has an equivalent in sunlight. The experiments herein reported indicate that sunlight does not contain at all or only to a very slight degree the equivalent of the anti-xerophthalmic factor in cod-liver oil."

Shortly after the publication of the researches cited, it became apparent to many observers that sunlight was not a substitute for vitamin D but merely a means of converting a precursor present in the body into the vitamin. The early work of Hume, and Goldblatt and Soames probably represents the first proof of the photosynthesis of vitamin D in the animal body and, in view of present knowledge, may be interpreted as the first demonstration of the absorption of vitamin D through the skin and the subsequent storage of this vitamin in the animal tissues. The absorption of vitamin D through the skin, which had been indicated from the beginning of the vast vitamin D problem, remained to be demonstrated conclusively by experiments on the direct topical application of the vitamin.

One of the early proofs that vitamin D can be absorbed through the skin is found in the work of Price[9] who, in 1926, conducted experiments in which he topically applied cod-liver oil to chicks, and as a result was able to advance conclusive

[8]Powers, G. F., Park, E. A., and Simmonds, N., *J. Biol. Chem.*, **55** (1923): 575.

[9]Price, W. A., *Ind. Eng. Chem.*, **18** (1926): 679.

proof that the vitamin from the cod-liver oil had been effectively absorbed through the skin. Following the work of Price similar results, confirming the earlier investigations, were reported by various researchers, notably among whom were Hume, Lucas and Smith,[10] Amrhein,[11] Astrowe and Morgen,[12] Fodor [13] and Schuler.[14]

Part I—Speed of Absorption of Vitamin D Through the Skin

In the researches cited in the literature the vitamin was allowed to remain in contact with the skin for prolonged intervals. The object of this present research was to determine if vitamin D is absorbed through the skin in quantities sufficient to cure rickets when allowed to remain in contact with the skin for only brief periods of time.

EXPERIMENTAL

Thirty-day-old white rats, weighing between 30 and 50 grams each, were used. The rats were fed the following rachitogenic diet, specified in the United States Pharmacopoeia, Eleventh Decennial Revision, 1936, except that 2 per cent non-irradiated yeast was substituted for a like amount of the yellow cornmeal:

Yellow cornmeal	74 per cent
Wheat gluten	20 per cent
Calcium carbonate	3 per cent
Sodium chloride	1 per cent
Non-irradiated yeast	2 per cent

[10]Hume, E. M., Lucas, N. S., and Smith, H. H., *Biochem. J.*, 21 (1927): 362.

[11]Amrhein, F. J., *J. Am. Pharm. Assoc.*, 22 (1934): 182.

[12]Astrowe, P. S., and Morgen, R. A., *Am. J. Diseases Children*, 49 (1935): 912.

[13]Fodor, M. E., *Z. Vitaminforsch.*, 3 (1934): 241.

[14]Schuler, R., *Drug Cosmetic Ind.*, 38 (1936): 485.

To this diet was added an amount of carotene in oil sufficient to supply the vitamin A requirements of the animals.

The animals were kept in screen-bottomed wire cages completely protected from sunlight. The only source of illumination was low-intensity, indirect light from incandescent lamps. Feed and water were present in the cages at all times. During the first 3 weeks the rats were kept in large cages in groups of 10 or 12. Each animal was weighed twice weekly and the weights recorded. At the end of the depletion period the rats were placed in individual cages for the duration of the test period.

Approximately 3 weeks after feeding the vitamin D-deficient diet, radiographs were taken to determine the extent to which the animals had developed rickets. The joint between the femur and the tibia of the left hind leg was used for this purpose. Radiographs were taken at 5-day intervals until most of the rats had developed severe rickets. The rats at this time were considered to be in proper condition for the treatment, i.e., the topical application of vitamin D.

Since there was no prescribed technic recommended by the United States Pharmacopoeia for the topical application of vitamins, after careful consideration of all the factors involved, the following procedure was adopted: The hair was clipped from an area of approximately 12 sq. cm. on the back of each rat close to the neck. 1453 U. S. P. units of vitamin D were incorporated in each 3.7-ounce bar of soap. The soap was dissolved in water so that 1 cc. of the solution contained 3 U. S. P. units of vitamin D. This vitamin D-containing medium was applied only to the limited clipped area on the back of each rat. The soap was allowed to remain in contact with the skin for periods ranging from 30 to 300 seconds and was then thoroughly removed by swabbing the skin with a piece of sterile terry cloth dipped in clear water. The area was then dried thoroughly with another piece of terry cloth.

Topical Applications 30 and 60 Seconds—Summer

In this experiment 38 animals were divided into 4 groups and treated as follows:

Group I. . . . .Negative Control (9 rats)

Group II. . . .3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 30 seconds (11 rats)

Group III. . .Negative Control (9 rats)

Group IV. . .3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 60 seconds (9 rats)

The negative control rats were washed for the same length of time as the test animals, with soap containing no vitamin D.

RESULTS

None of the negative control rats, Group I, showed healing of rickets either at the end of the 2-week test period or at the end of the 4-week test period.

The animals in Group II, which received 3 U. S. P. units of vitamin D by topical application for 30 seconds twice daily, showed almost the same negative results as Group I, except that at the end of the 4-week test period there was definite healing in 27 per cent of the animals. The remaining 63 per cent developed increased rickets.

None of the negative control rats in Group III showed healing of rickets at the end of the first 10 days, and at the end of the third week 8 of the 9 rats were severely rachitic, the ninth showing moderate rickets.

Group IV, to which 3 U. S. P. units of vitamin D in a soap medium were applied for 60 seconds twice daily, failed to show any healing of rickets at the end of 10 days, while at the end of 3 weeks all had developed very severe rickets.

X-Ray Diagnoses for Rickets

TABLE I

Group I— Negative Control

Soap containing no vitamin D topically applied twice daily for periods of 30 seconds

	Degree of Rickets		
	May 21	June 3	June 17
Rat No. 1	Severe	Very severe	Very severe
2	Slight	Moderate	Severe
3	Moderate	Severe	Very severe
4	Moderate	Very severe	Very severe
5	Moderate	Severe	Very severe
6	Moderate	Moderate	Severe
7	Moderate	Severe	Very severe
8	Moderate	Severe	Very severe
9	Moderate	Severe	Severe

Group II—3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 30 seconds

	Degree of Rickets		
	May 21	June 3	June 17
Rat No. 1	Moderate	Moderate	Slight
2	Severe	Very severe	Very severe
3	Severe	Moderate	Moderate
4	Moderate	Severe	Severe
5	Very severe	Moderate	Moderate
6	Moderate	Severe	Very severe
7	Moderate	Very severe	Very severe
8	Moderate	Very severe	Very severe
9	Severe	Severe	Very severe
10	Moderate	Severe	Very severe
11	Severe	Very severe	Very severe

X-Ray Diagnoses for Rickets

TABLE I—Continued

Group III— Negative Control

Soap containing no vitamin D topically applied twice daily for periods of 60 seconds

	Degree of Rickets		
	July 9	July 19	July 29
Rat No. 1	Severe	Very severe	Very severe
2	Moderate	Severe	Very severe
3	Slight	Severe	Very severe
4	Severe	Very severe	Very severe
5	Slight	Slight	Moderate
6	Moderate	Severe	Very severe
7	Severe	Very severe	Very severe
8	Moderate	Severe	Very severe
9	Slight	Moderate	Very severe

Group IV—3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 60 seconds

	Degree of Rickets		
	July 9	July 19	July 29
Rat No. 1	Moderate	Severe	Very severe
2	Moderate	Severe	Very severe
3	Moderate	Severe	Very severe
4	Severe	Very severe	Very severe
5	Severe	Very severe	Very severe
6	Moderate	Severe	Very severe
7	Moderate	Very severe	Very severe
8	Moderate	Moderate	Very severe
9	Moderate	Severe	Very severe

As will be noted from the dates of the X-ray diagnoses, these experiments were conducted during the summer months, the first set during May and June, the second during June and July. Previous preliminary experiments under similar conditions yielded positive results, making it difficult to explain the negative results obtained in this experiment. In the work of Tourtelotte and Bacon[15] it is shown that the curing of rickets is characterized by decided seasonal variations. They found as much as 400 per cent variation in the reaction of the animals, which they deemed due mainly to change in temperature, humidity and like factors.

In view of these results it occurred to us that possibly seasonal variations were responsible for the discrepancies in our results, and therefore the experiments were repeated during the winter.

Topical Application 60 *Seconds—Winter*

During the following winter months 29 animals were divided into 2 groups and treated as follows:

Group I. . . . .Negative Control (11 rats)

Group II. . . .3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 60 seconds (18 rats)

RESULTS

The negative control rats, Group I, at the end of the 2-week test period, showed no signs of healing in approximately 64 per cent of the animals, while in 36 per cent there was a very slight, yet noticeable, improvement in the rachitic condition.

Of the 18 rats in Group II, which received topical applications of 3 U. S. P. units of vitamin D in a soap medium for

[15]Tourtelotte, D., and Bacon, W. E., *J. Nutrition*, **10** (1935): 683.

X-Ray Diagnoses for Rickets

TABLE II

Group I— Negative Control

Soap containing no vitamin D topically applied twice daily for periods of 60 seconds

	Degree of Rickets	
	Dec. 8	Dec. 16
Rat No. 1	Severe	Severe
2	Very severe	Very severe
3	Very severe	Very severe
4	Very severe	Severe
5	Very severe	Very severe
6	Very severe	Very severe
7	Very severe	Very severe
8	Very severe	Moderate
9	Very severe	Severe
10	Very severe	Severe
11	Very severe	Very severe

Group II—8 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 60 seconds

	Degree of Rickets	
	Dec. 8	Dec. 16
Rat No. 1	Very severe	Mild
2	Very severe	Mild
3	Very severe	Mild
4	Very severe	Mild
5	Very severe	Slight
6	Very severe	Severe
7	Very severe	Severe
8	Very severe	Moderate
9	Very severe	Mild
10	Very severe	Moderate
11	Very severe	Severe
12	Very severe	Very severe
13	Very severe	Moderate
14	Very severe	Moderate
15	Very severe	Moderate
16	Very severe	Very severe
17	Very severe	Moderate
18	Very severe	Very severe

periods of 60 seconds twice daily, 66 per cent showed definite healing of rickets, 17 per cent showed slight, and 17 per cent showed no healing of rickets.

This experiment was conducted during the months of November and December.

Topical Application 60 Seconds—Winter Second Group of Experiments

Another set of experiments was conducted shortly after the one just reported.

The animals were divided into 2 groups and treated as follows:

Group I. . . .Negative Control (11 rats)

Group II. . .3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 60 seconds (11 rats)

RESULTS

Ten of the 11 rats (or more than 90 per cent) in the negative control group (Group I) showed no healing of rickets; the eleventh rat showed but slight healing.

Of the rats in Group II, to which 3 U. S. P. units of vitamin D contained in soap were topically applied twice daily for periods of 60 seconds, 45 per cent showed slight or no healing. The remaining 55 per cent showed pronounced healing of rickets.

The rats used in this experiment were slightly older than recommended by the United States Pharmacopoeia; hence, these results, although conclusive from a practical standpoint, cannot of themselves be considered sufficient from a theoretical standpoint.

X-Ray Diagnoses for Rickets

TABLE III

Group I— Negative Control, no soap applied

	Degree of Rickets	
	Dec. 18	Dec. 31
Rat No. 1	Very severe	Very severe
2	Very severe	Very severe
3	Severe	Severe
4	Severe	Severe
5	Very severe	Very severe
6	Very severe	Severe
7	Very severe	Very severe
8	Very severe	Very severe
9	Very severe	Very severe
10	Severe	Severe
11	Very severe	Very severe

Group II—3 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 60 seconds

	Degree of Rickets	
	Dec. 18	Dec. 31
Rat No. 1	Severe	Severe
2	Severe	Severe
3	Moderate	Moderate
4	Severe	Severe
5	Moderate	Slight
6	Very severe	Severe
7	Very severe	Moderate
8	Very severe	Moderate
9	Moderate	Moderate
10	Severe	Moderate
11	Very severe	Moderate

Topical Application 120, 180 *and* 300 *seconds—Fall*

In the following experiment the time of application was increased in order to demonstrate the speed of absorption of vitamin D through the skin when applied topically in a soap medium.

Thirty-seven animals were divided into 4 groups and treated as follows:

Group I. . . . .Negative Control (10 rats)

Group II. . . . .4 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 120 seconds (11 rats)

Group III. . .4 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 180 seconds (11 rats)

Group IV. . .4 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 300 seconds (5 rats)

RESULTS

At the end of the 4-week test period the negative control rats, Group I, were diagnosed by X-rays and all showed increased and very severe rickets.

Of the rats in Group II, to which 4 U. S. P. units of vitamin D in soap were topically applied twice daily for periods of 120 seconds, approximately 81 per cent showed pronounced healing of rickets; the remaining 19 per cent failed to show healing.

Of Group III, to which 4 U. S. P. units of vitamin D in soap were topically applied twice daily for periods of 180 seconds, 63 per cent showed definite healing, while the remaining 37 per cent, although showing no healing, gave no evidence of increased rickets.

Group IV consisted of 5 rats to which 4 U. S. P. units of vitamin D in soap were topically applied twice daily for

X-Ray Diagnoses for Rickets

TABLE IV

Group I— Negative Control

	Degree of Rickets	
	Sept. 29	Oct. 27
Rat No. 1	Severe	Very severe
2	Moderate	Very severe
3	Moderate	Very severe
4	Moderate	Very severe
5	Moderate	Very severe
6	Moderate	Very severe
7	Moderate	Very severe
8	Moderate	Very severe
9	Severe	Very severe
10	Severe	Very severe

Group II—4 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 120 seconds

	Degree of Rickets	
	Sept. 29	Oct. 27
Rat No. 1	Very severe	Slight
2	Severe	Moderate
3	Moderate	No rickets
4	Very severe	Slight
5	Severe	Moderate
6	Severe	Very severe
7	Very severe	Moderate
8	Severe	Severe
9	Severe	Moderate
10	Severe	Slight
11	Very severe	Severe

X-Ray Diagnoses for Rickets

TABLE IV—Continued

Group III—4 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 180 *seconds*

	Degree of Rickets	
	Sept. 29	Oct. 27
Rat No. 1	Very severe	Moderate
2	Very severe	Very severe
3	Very severe	Very severe
4	Very severe	Very severe
5	Very severe	Moderate
6	Very severe	Moderate
7	Severe	Slight
8	Very severe	Slight
9	Very severe	Severe
10	Very severe	Very severe
11	Very severe	Severe

Group IV—4 U. S. P. units of vitamin D in soap topically applied twice daily for periods of 300 *seconds*

	Degree of Rickets	
	Oct. 12	Oct. 27
Rat No. 1	Very severe	Moderate
2	Very severe	Slight
3	Very severe	Slight
4	Very severe	Slight
5	Very severe	Slight

periods of 300 seconds. These rats were very severely rachitic at the beginning of the test period, but at the end of 15 days 100 per cent showed almost complete healing.

This series of experiments was conducted during the months of September and October.

On Plates I and II are prints of the radiographs taken at the beginning and end of the 15-day test period of the rats in Group IV, Table IV, together with a typical negative control from Group I, Table IV.

CONCLUSIONS

Vitamin D is absorbed through the skin with extreme rapidity, some cases showing marked results in intervals as low as 60 seconds.

When only 4 U. S. P. units of vitamin D are allowed to remain in contact with the skin for periods of 5 min., twice daily, the absorption is sufficient to almost completely cure severe rickets in rats in 15 days.

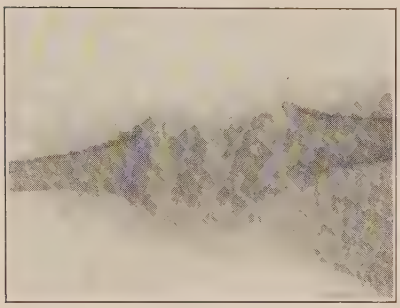
The absorption of vitamin D through the skin apparently takes place at a slower rate during the summer months than in the winter months.

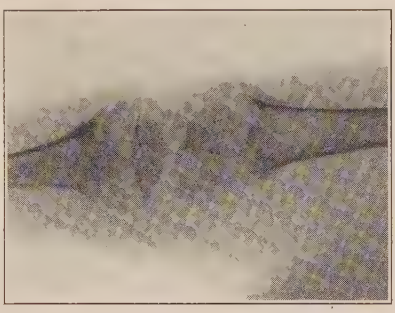
PLATE I

Radiographs showing the effect of the absorption of 4 U. S. P. units of vitamin D through the skin when applied topically in soap for 300 seconds twice daily. (Group IV, Table IV)

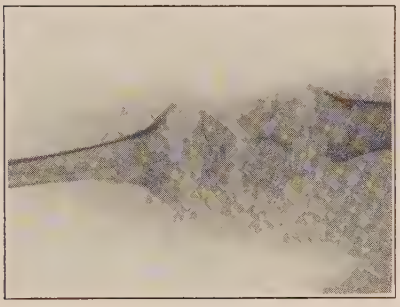
Before Treatment

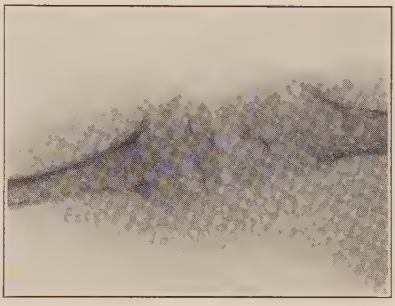
After Treatment



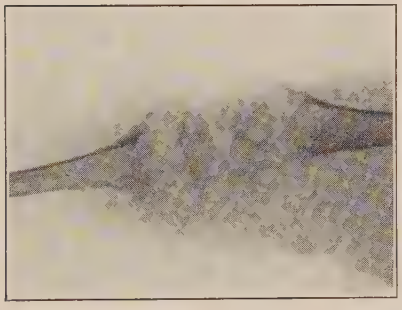


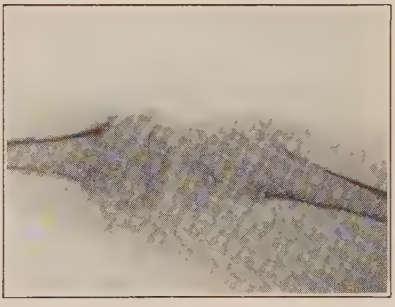
Rat No. 1





Rat No. 2





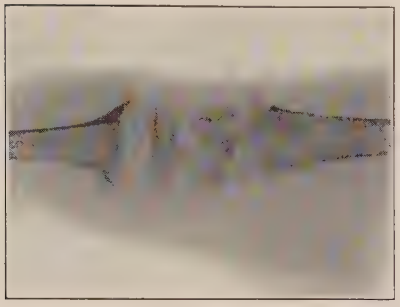
Rat No. 3

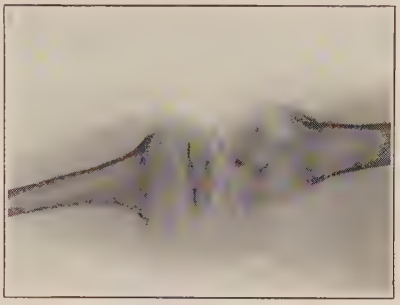
PLATE II

Radiographs showing the effect of the absorption of 4 U. S. P. units of vitamin D through the skin when applied topically in soap for 300 seconds twice daily. (Group IV, Table IV)

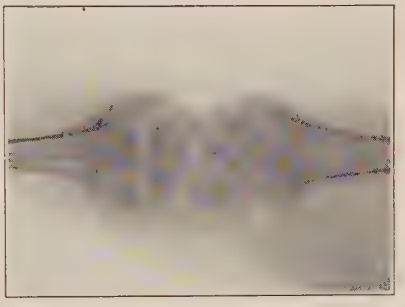
Before Treatment

After Treatment

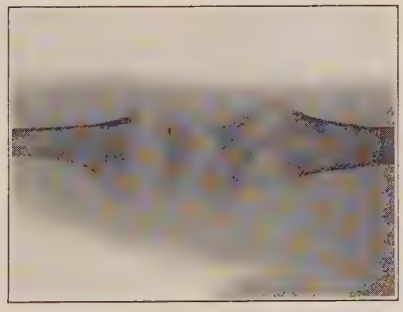




Rat No. 4



Rat No. 5



Typical Negative Control Rat
(Group I, Table IV)

THE ABSORPTION OF VITAMIN D THROUGH THE SKIN*

By AGNES C. HELMER and REV. CORNELIUS H. JANSEN

Part II—Efficiency of Absorption of Vitamin D Through the Skin

A great deal has been written concerning the absorption of vitamin D through the skin but very little has been written regarding the *efficiency* of this type of absorption. There are those who still maintain that nothing can penetrate the unbroken skin, but this has been so thoroughly disproved that it need be given no further consideration in this paper.

Recently Fodor[16] and also Schuler[17] reported that the quantity of vitamin D necessary when topically applied is about 10 times that required when administered orally.

The purpose of this research is to establish more definitely the relationship which exists between the absorption of vitamin D through the skin and through the digestive tract.

In Part I it has been shown that the amount of vitamin D needed to cure rickets depends on seasonal variations, probably temperature changes. By using several groups of animals, kept at different temperatures, the cause of this variation might be more definitely determined.

Albino rats, weighing approximately 40 grams each and about 30 days old, were used in this series of experiments. The rats were separated into 2 divisions of 41 rats each. One

*This research was conducted under The Andrew Jergens Fellowship, Cincinnati, Ohio.

[16]Fodor, M. E., *loc. cit.*

[17]Schuler, R., *loc. cit.*

division was treated at an average temperature of 90° F. and the other at an average temperature of 70° F.

The animals were numbered, weighed and each division separated into 4 smaller groups. They were housed in large cages and fed the vitamin D-deficient diet* for 3 weeks, during which time they were weighed twice a week.

After the depletion period the rats were X-rayed to determine the degree of rickets and were then placed in individual cages and treated as follows:

Rats kept at approximately 90° F.

Group I. . . . .Negative Control

Group II. . . . *Viosterol applied topically*; 0.5 U. S. P. unit of vitamin D per day

Group III. . . *Viosterol applied topically*; 1 U. S. P. unit of vitamin D per day

Group IV. . . . *Viosterol fed*; 0.5 U. S. P. unit of vitamin D per day

Rats kept at approximately 70° F.

Group V. . . . .Negative Control

Group VI. . . . *Viosterol applied topically*; 0.5 U. S. P. unit of vitamin D per day

Group VII. . . *Viosterol applied topically*; 1 U. S. P. unit of vitamin D per day

Group VIII. . *Viosterol fed*; 0.5 U. S. P. unit of vitamin D per day

One gram of the viosterol used contained 10,000 U. S. P. units of vitamin D. The viosterol was diluted with olive oil so that 0.1 cc. contained 1 U. S. P. unit of the vitamin.

The oil was applied to the back of the neck of some rats and fed by mouth to others, according to the group in which

*Diet given in Part I.

they were placed. The oil was rubbed into the skin until all of it had been absorbed. After a 2-week treatment period, X-rays were taken to determine the curative effect of viosterol when applied topically as compared with the efficacy of vitamin D as a curative agent when taken orally.

RESULTS

Rats kept at approximately 90° F.

At the end of the depletion period, X-rays disclosed that 90 per cent of the rats in the four groups had developed severe, or very severe, rickets; 5 per cent moderate rickets. The remaining 5 per cent had died before the X-rays were taken.

At the end of the test period the negative control rats, Group I, were diagnosed as very severely rachitic in 100 per cent of the cases.

All the animals in Group II, to which 0.05 cc. of viosterol containing 0.5 U. S. P. unit of vitamin D was applied topically once a day, showed pronounced healing of rickets.

All the rats in Group III, to which 0.1 cc. of viosterol containing 1 U. S. P. unit of vitamin D was applied topically once a day, showed pronounced healing of rickets; 40 per cent showing complete healing and 60 per cent almost complete healing.

With the exception of 1 rat which died, 100 per cent of the rats in Group IV, which were daily fed 0.05 cc. viosterol containing 0.5 U. S. P. unit of vitamin D, showed pronounced healing of rickets. Thirty-three per cent showed complete healing of rickets, and 67 per cent almost complete healing.

X-Ray Diagnoses for Rickets

TABLE V

Rats Kept at Approximately 90° F.

Group I— Negative Control

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Very severe	Very severe
2	Very severe	Very severe
3	Very severe	Very severe
4	Very severe	Very severe
5	Very severe	Very severe
6	Very severe	Very severe
7	Severe	Very severe
8	Very severe	Very severe
9	Very severe	Very severe
10	Very severe	Very severe

Group II—Viosterol applied topically; 0.5 *U. S. P. unit of vitamin D daily*

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Severe	Slight
2	Very severe	Moderate
3	Very severe	Severe
4	Very severe	Moderate
5	Very severe	Slight
6	Very severe	Moderate
7	Severe	Slight
8	Dead	
9	Dead	
10	Severe	Moderate
11	Very severe	Severe

X-Ray Diagnoses for Rickets

TABLE V—Continued

Rats Kept at Approximately 90° F.

Group III—Viosterol applied topically; 1 *U. S. P. unit of vitamin D daily*

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Very severe	No rickets
2	Severe	Slight
3	Severe	Slight
4	Moderate	No rickets
5	Very severe	Moderate
6	Very severe	Slight
7	Very severe	Moderate
8	Severe	No rickets
9	Moderate	No rickets
10	Very severe	Slight

Group IV—Viosterol fed; 0.5 *U. S. P. unit of vitamin D daily*

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Severe	Slight
2	Severe	No rickets
3	Very severe	Slight
4	Severe	Moderate
5	Severe	Slight
6	Severe	No rickets
7	Severe	Slight
8	Severe	Dead
9	Severe	No rickets
10	Very severe	Slight

RESULTS

Rats kept at approximately 70° F.

At the end of the depletion period, X-rays showed that 71 per cent of the rats in this division had severe or very severe rickets, and 27 per cent had slight or moderate rickets. Two per cent of the rats were dead.

The negative control rats, Group V, diagnosed by means of X-rays at the end of the 2-week test period, were found to have more severe rickets in 60 per cent of the cases, and 40 per cent showed slight healing.

One hundred per cent of the rats in Group VI, to which 0.05 cc. viosterol containing 0.5 U. S. P. unit of vitamin D was applied topically once a day, showed pronounced healing of rickets; 40 per cent showing complete healing, the remaining 60 per cent almost complete healing.

Group VII, to which 0.1 cc. viosterol containing 1 U. S. P. unit of vitamin D was applied topically once a day, showed pronounced healing of rickets in 100 per cent of the cases; 40 per cent being completely cured and 60 per cent almost completely cured.

In Group VIII, which was fed 0.05 cc. viosterol containing 0.5 U. S. P. unit of vitamin D daily, there was 100 per cent pronounced healing of rickets with 20 per cent completely cured and 80 per cent almost completely cured.

X-Ray Diagnoses for Rickets

TABLE VI

Rats Kept at Approximately 70° F.

Group V—Negative Control

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Very severe	Very severe
2	Very severe	Very severe
3	Severe	Moderate
4	Moderate	Moderate
5	Very severe	Very severe
6	Very severe	Moderate
7	Very severe	Moderate
8	Moderate	Slight
9	Moderate	Moderate
10	Slight	Slight

Group VI—Viosterol applied topically; 0.5 *U. S. P. unit of vitamin D daily*

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Slight	Slight
2	Slight	No rickets
3	Moderate	No rickets
4	Severe	Slight
5	Very severe	Moderate
6	Very severe	Slight
7	Moderate	No rickets
8	Moderate	No rickets
9	Moderate	Slight
10	Dead	
11	Very severe	Slight

X-Ray Diagnoses for Rickets

TABLE VI—Continued

Rats Kept at Approximately 70° F.

Group VII—Viosterol applied topically; 1 U. S. P. unit of vitamin D daily

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Very severe	Slight
2	Very severe	Slight
3	Very severe	Slight
4	Severe	No rickets
5	Very severe	Moderate
6	Very severe	No rickets
7	Very severe	No rickets
8	Very severe	Slight
9	Very severe	Slight
10	Very severe	No rickets

Group VIII—Viosterol fed; 0.5 U. S. P. unit of vitamin D daily

	Degree of Rickets	
	July 21	August 3
Rat No. 1	Very severe	Moderate
2	Severe	Moderate
3	Slight	No rickets
4	Very severe	Slight
5	Very severe	Moderate
6	Very severe	No rickets
7	Very severe	Moderate
8	Very severe	Moderate
9	Very severe	Slight
10	Very severe	Moderate

On Plates III and IV are shown radiographs of four rats used to compare the absorption of vitamin D through the skin with the absorption through the digestive tract.

The upper photographs of Plate III are those of a negative control rat and indicate very severe rickets both at the beginning and the end of the experiment. The lower photographs are of a rat fed 0.5 U. S. P. vitamin D unit (viosterol). At the beginning of the test period the X-ray shows very severe rickets, while the X-ray taken at the end of the test period indicates almost complete cure of rickets.

On Plate IV the upper photographs are of a rat to which 0.5 U. S. P. vitamin D unit was topically applied. The X-ray shows severe rickets at the beginning of the test period, and only slight rickets at the end of the treatment. The lower photographs are of a rat to which 1 U. S. P. vitamin D unit was topically applied. They reveal very severe rickets before treatment and slight rickets after treatment.*

CONCLUSIONS

The degree of rickets *developed* by animals seems to be dependent, to at least a slight degree, upon the temperature at which they are housed, animals maintained at a higher temperature showing a greater degree of rickets than those maintained at a lower temperature.

The temperature at which experimental animals are treated is a factor which must be considered in determining the amount of vitamin D necessary for the *cure* of rickets when the vitamin is topically applied. This is not surprising in view of the fact that increased doses of vitamin D are necessary in oral administrations during the summer months.

*These experiments have all been repeated using cream and lotion as a carrier of the vitamin with equally conclusive results.

The efficiency of the absorption of vitamin D through the skin seems to be approximately equal to the efficiency of the absorption of vitamin D through the digestive tract.

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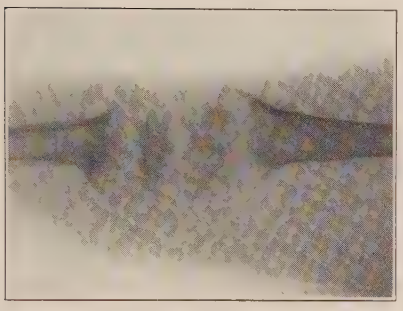
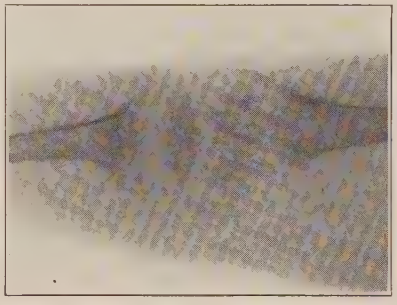
PLATE III

Typical radiographs showing the comparison between the absorption of vitamin D through the skin and through the digestive tract.

Negative control Rat.

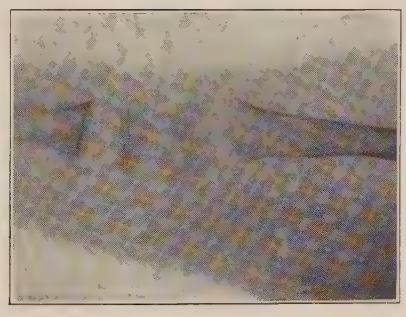


Beginning of Test Period

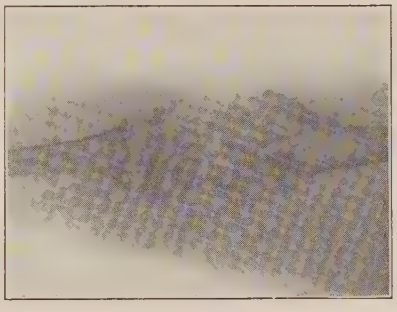


End of Test Period

Rat fed 0.5 *U. S. P. vitamin D unit (Viosterol) daily.*



Before Treatment

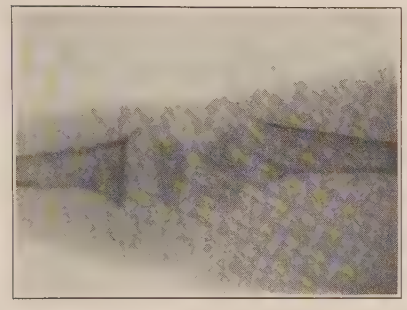


After Treatment

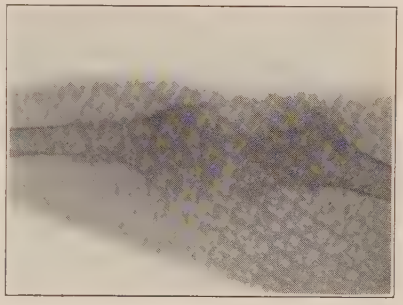
PLATE IV

Typical radiographs showing the comparison between the absorption of vitamin D through the skin and through the digestive tract.

Rat to which 0.5 *U. S. P. vitamin D unit (Viosterol) was topically applied daily.*

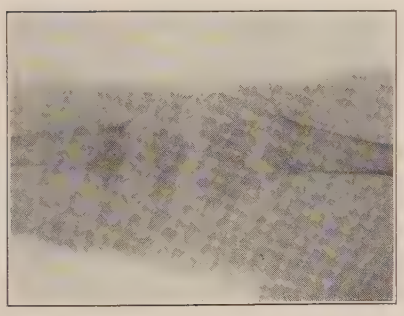


Before Treatment

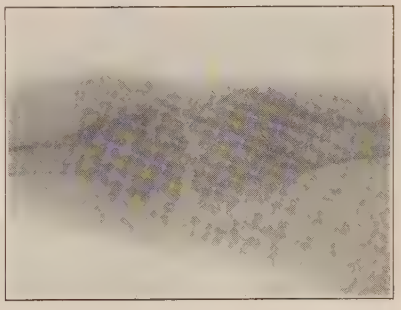


After Treatment

Rat to which 1 *U. S. P. vitamin D unit (Viosterol) was topically applied daily.*



Before Treatment



After Treatment

THE DEVELOPMENT OF PLANT PIGMENTS IN SEEDLINGS GROWN IN THE DARK

By WILLIAM A. BECK, S.M.*

It was important to determine the amount of pigment which developed in seedlings not exposed to light in order to obtain the necessary data for the determination of pigments which developed under the influence of certain imposed physical factors.

Threshold values had to be obtained so that the control plants taken from the dark chamber, where they were sprouted, might be a more exact standard with which the pigment development of experimental plants could be compared. It was, of course, interesting to know if any or all of the three pigments (chlorophyll, xanthophyll and carotene) developed and, if so, at what rate. Once these facts were determined the approximate amount of pigment present in a control plant could easily be calculated provided its age was known and it had not been exposed to light.

EXPERIMENTAL

Sunflowers (*helianthus annuus*) were used. Only the cotyledons were taken, since the stems were white and contained practically no pigments. The plants were grown in small pots sunk in German peat and filled with soil prepared by mixing 50 per cent of light green-house soil with 50 per cent German peat. At the time of planting, the soil was moistened with Knopp's solution. No water was added from the time of planting to the time of testing. The surrounding atmosphere was constantly saturated with moisture (100 per cent relative humidity). The temperature was practically constant at 75° F.

*Lecturing Professor, University of Dayton, Dayton, Ohio.

Willstaetter's method as modified by Schertz[1] was used for extracting the pigments. A photodensitometer[2] was employed to determine quantitatively the amount of pigment present, which was measured as a function of the light absorbed when rays were passed through a cell containing the pigment in solution. From the appearance of the cotyledons, it was evident that no chlorophyll developed in the dark, but the complete procedure for extraction and separation was always carried out. In all cases the "brown phase" in the process of saponification was reached but no trace of chlorophyllin appeared. It is significant that the brown solution always appeared in place of chlorophyllin, which probably represented the "leukophyll" of Liro,[3] the "chlorophyllogen" of Palladin, or the "protochlorophyll" of Eyster.[4]

The substance, which probably is formed in the dark and quickly changes into chlorophyll when the plant is exposed to light, has never been isolated, but there is considerable evidence that it actually exists. The extract from etiolated leaves shows the characteristic absorption bands between 6,200 Å and 6,400 Å. The substance seems to be a colorless progenitor of chlorophyll. It acts as an autocatylitic agent that can quickly produce chlorophyllogen with the aid of light.[5] The material which invariably appeared in the author's experiments as a brown material after the pigments were saponified in the regular manner, could not have been flavones since care was always taken to remove them. The findings agree with the accepted view that chlorophyll cannot form in phanerogams except in the presence of light, even though it has been found that it can form without the aid of light in certain algae and certain sprouting conifers.[6]

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[1]Willstaetter-Schertz, Investigations on Chlorophyll (1927).

[2]Beck, W. A., *Science*, 85 No. 2206 (1937): 368.

[3]Benecke-Jost, *Pflanzenphysiol.*, 1 (1924): 209.

[4]Heinricher, E., *Ber. Bot. Ges.*, 1 (1883): 433.

[5]Benecke-Jost, *loc. cit.*

[6]Benecke-Jost, *loc. cit.*; Henricher, *loc. cit.*; Schimper, A. F., *Jahrb. Wiss. Bot.* 16 (1885): 1246; Schmidt, A., *Beitr. z. Biol.*, 12 (1914): 269.

TABLE I

The Amount of Carotinoid Pigments Developed in the Dark

Age in Days	No. of Cotyledons	Grams of Wet Weight	Mg. per 100 g. of Wet Weight		Mg. per 1000 Cotyledons		Mg. per 5 g. of pulp		$\frac{\text{Xanthophyll per g.}}{\text{Carotene per g.}} = R$
			Xanthophyll	Carotene	Xanthophyll	Carotene	Xanthophyll	Carotene	
4	62	4.2	1.5	0.17	1.04	0.11	0.84	0.09	9.0
5	62	4.3	4.4	0.52	3.2	0.36	2.4	0.27	8.5
6	48	3.4	6.3	0.55	4.25	0.39	5.0	0.44	11.4
7	56	4.5	7.2	0.55	5.8	0.44	5.5	0.415	13.0
14	60	6.1	9.2	0.45	9.4	0.458	8.91	0.415	20.0

DISCUSSION

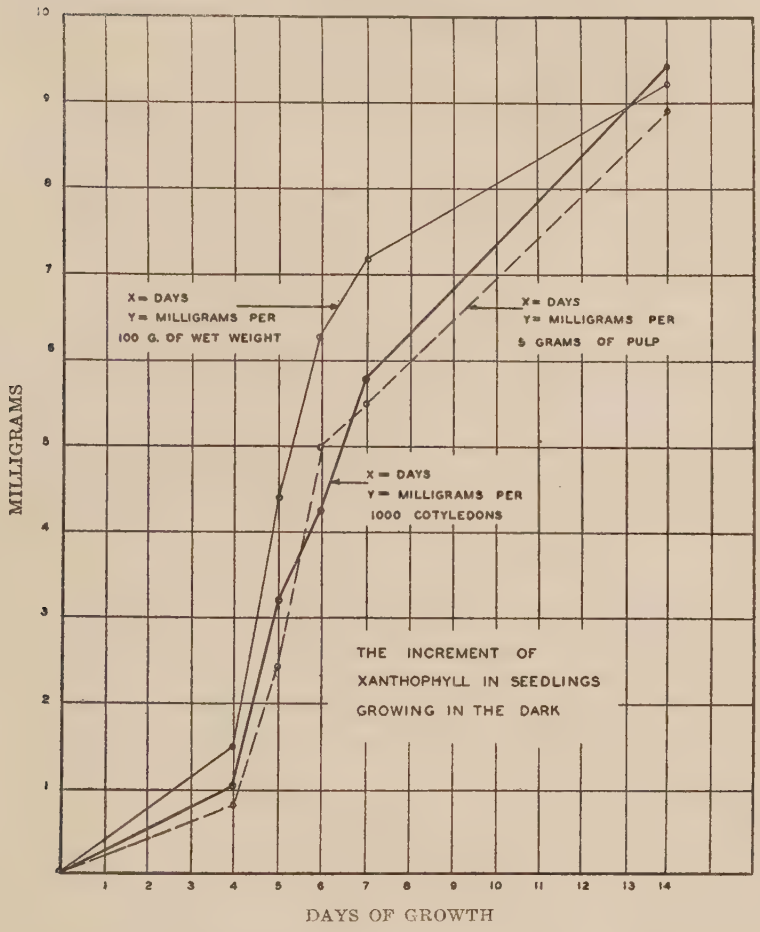
The amount of pigment present is often expressed as a function of the wet weight. These values are given in the Table. Since the amount of water taken up by the plant during the period of growth need not be in keeping with the development of the pigment, this method of expression does not seem to be advisable.

The amount of pigment present may be expressed as a function of the amount of pulp. The corresponding values are given in the Table. The figures make it clear that the amount of pulp which developed was by no means proportional to the water absorbed. This fact is particularly evident from the experiment with the 4-day-old plants and the 14-day-old plants. In the case of the 4-day-old, 62 cotyledons were used; they had a wet weight of 4.2 g. and yielded 0.384 g. of pulp, while 60 cotyledons of the 14-day-old plants had a wet weight of 6.1 g. and yielded only 0.3165 g. of pulp.

If it be assumed that the cotyledons have the same initial average size and equal capacity for developing pigment, an expression of the amount of pigment per fixed number of cotyledons should give the most satisfactory way of expressing the increment in pigment. The corresponding values are given in the Table. These values are not dependent on any other developing parts in the plants.

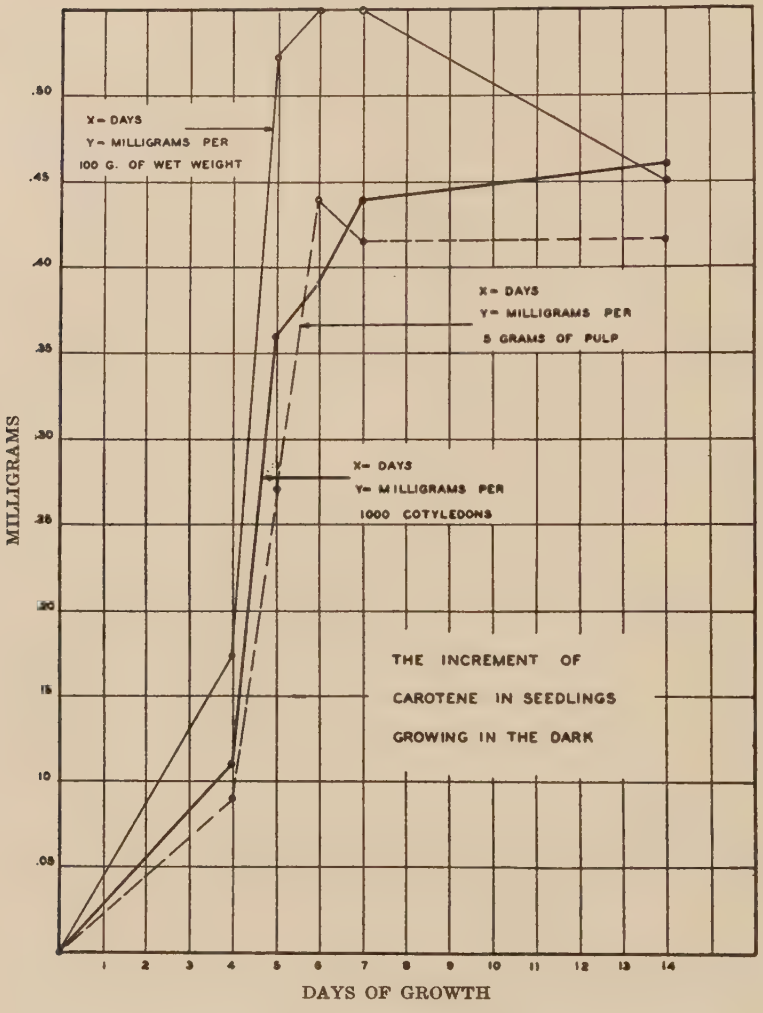
The relation between the rate of development of one pigment and the other can be explained in terms of the ratio between the amount of xanthophyll and the amount of carotene present under the same conditions. These ratios are given in the last column of the Table. From these ratios it is evident that the xanthophyll develops much faster than does the carotene. The formation of xanthophyll as a function of time is shown graphically in Fig. 1. The amount of pigment is expressed in three ways, viz., per unit of wet weight, per cotyledon and per unit of pulp.

FIG. 1—*Development of Xanthophyll in Seedlings Grown in the Dark*



Carotene formation as a function of time is shown graphically in Fig. 2. From the Table it may be seen that the rate of formation of carotene decreases rapidly after the fifth day. Although the carotene formation after this time is very small, the amount per cotyledon steadily increases. If the rate of

FIG. 2—*Development of Carotene in Seedlings Grown in the Dark*



formation of carotene per unit of wet weight is studied as a function of time the amount of carotene decreases after having remained constant for some time.

SUMMARY

1. Sunflower seedlings were grown in the dark. The amount of pigment present in the cotyledons was determined for plants of different ages.

2. No chlorophyll developed, but there was evidence that a colorless substance which might be called protochlorophyll was present. The quantity was not determined.

3. Xanthophyll and carotene developed with age. This is best seen from the graphs in which the quantities of pigments developed per 1000 cotyledons were plotted as functions of the age of the cotyledons. The development of these pigments with age was not as evident when the amounts were expressed as functions of the wet weight or of the pulp. The rate of pigment development decreased with time. The xanthophyll developed more rapidly than did the carotene. The amount of carotene was always comparatively small.

CONCLUSIONS

These experiments make it evident that if sunflower seedlings are used to study the development of xanthophyll and carotene under different conditions of illumination, the formation of these pigments in the dark is a factor which must be considered.

Xanthophyll and carotene increase with the age of the plant, even though the plant is grown in the dark. The rate of development is not constant but decreases with time. Although no chlorophyll is formed, a colorless substance which may be protochlorophyll is present in seedlings grown in the dark.

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PHOTOTROPIC RESPONSE OF DROSOPHILA MELANOGASTER AND ITS MUTANTS

By JOHN C. FARDON, SISTER M. JORDAN CARROLL, O.P., and REV. WILLIAM A. SULLIVAN, O.P.

Phototropic response has long been observed among the insecta. Carpenter[1] (1905, 1908), Payne[2] (1911), Mast[3] (1911, 1917), Lutz[4] (1914), and McEwen[5] (1918) made studies of the reaction of organisms to light. McEwen, who did extensive work in this field with *Drosophila ampelophila*, concluded that the flies must be in a certain physiological state brought on by activity or at least accompanied by stimulation other than light before they are phototropic. He noted also that females react to light somewhat more readily than do males. This difference is more marked in young flies and steadily decreases until it is hardly noticeable when the flies have reached 8 or 9 days of age. He stated the time of maximum activity for both sexes to be at the age of 3 to 5 days. Using colored liquid filters, McEwen found that all the flies tested were most responsive to the violet light. Violet, green and red was the order of effectiveness of white, eosin, tinged and vermilion flies. However, wild and sepia flies were found to be more responsive to the red than the green.

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[1]Carpenter, F. W., *Am. Naturalist*, 39 (1905): 157; *J. Comp. Neurol.*, 18 (1908): 483.

[2]Payne, F., *Biol. Bull.*, 21 (1911): 297.

[3]Mast, S. O., Light and Behavior of Organisms, New York (1911); *J. Exp. Zool.*, 22 No. 3 (1917): 471.

[4]Lutz, F. E., *J. N. Y. Entomol. Soc.*, 22 No. 2 (1914): 134.

[5]McEwen, R. S., *J. Exp. Zool.*, 25 (1918): 49.

Since, in general, biological material presents extreme lack of constancy in its activity, it is patent that experimental data concerning this activity are often likely to be in error. Carefully measured stimuli applied to a given species often produce effects and responses of dissimilar, and occasionally of entirely opposite, characteristics.

In the work undertaken in this laboratory with *Drosophila melanogaster* and especially in the study of the response of this species to light, it has been observed that variations in response greatly limit the accuracy of the results obtained. At times there are great differences in the response curves of the same type of flies, of different types, and of the different sexes among a given type.

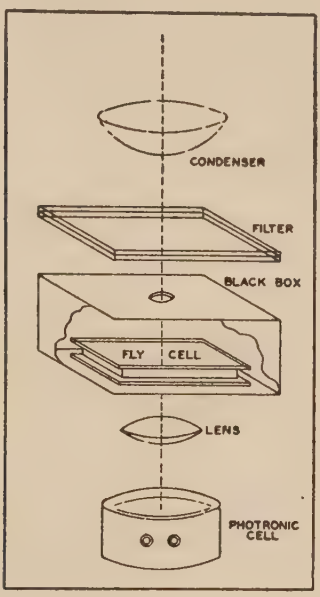
As a result of the irregularities in the response of the flies, it was necessary to conduct many preliminary investigations in order to determine the limits of experimental error. For example, before valid conclusions could be drawn it was necessary to answer such questions as the following: Do males and females differ in response? Do all flies of the same sex respond alike? Do flies differ in response over a passage of time? In other words, what are the factors governing the activities of flies?

EXPERIMENTAL

Our early experiments were conducted with a simple set-up such as shown diagrammatically in Fig. 1 except that the Photronic cell was replaced by a white screen.

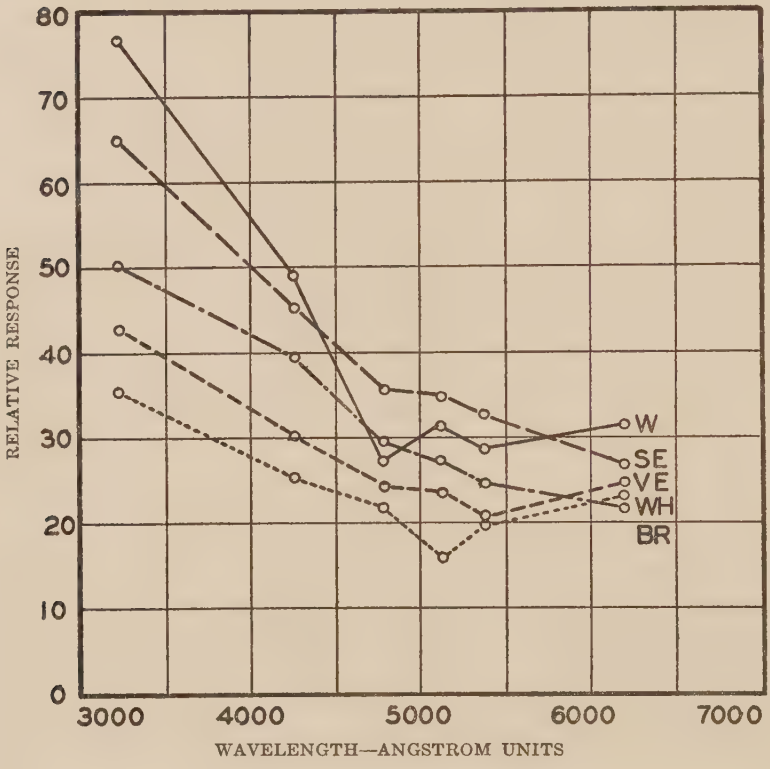
The flies were placed in a glass cell about 2 in. in diameter and $\frac{1}{8}$ in. deep. This cell was inserted in a dark chamber with two $\frac{1}{4}$-inch holes opposite each other. A condensing lens and a filter holder were placed above the chamber and a small lens below the chamber to focus the aperture and flies upon a white screen on the table. Twenty-five flies were then placed in the cell and the light turned on for 1 min.

FIG. 1—*Response Measuring Device*



The number of flies entering or passing the illuminated field was then counted. The flies were given 1-minute intervals of rest in total darkness between readings. Eastman Wratten filters, Series "M," Nos. 29 (red), 22 (orange), 58 (green), 45 (blue), and 35 (violet) were chosen for the colors. No corrections were made in these preliminary experiments for differences in filter densities. Since the response of the flies to the blue and violet was so pronounced, it was found extremely difficult to count the flies accurately by this method. The apparatus was then slightly modified to accommodate a Weston foot-candle meter or Photronic cell (Fig. 1). A 50-candle-power automobile lamp was used as the source of illumination. It was found necessary to operate the lamp from a storage battery in order to obtain a non-fluctuating current. The empty glass cell was inserted into the dark chamber and

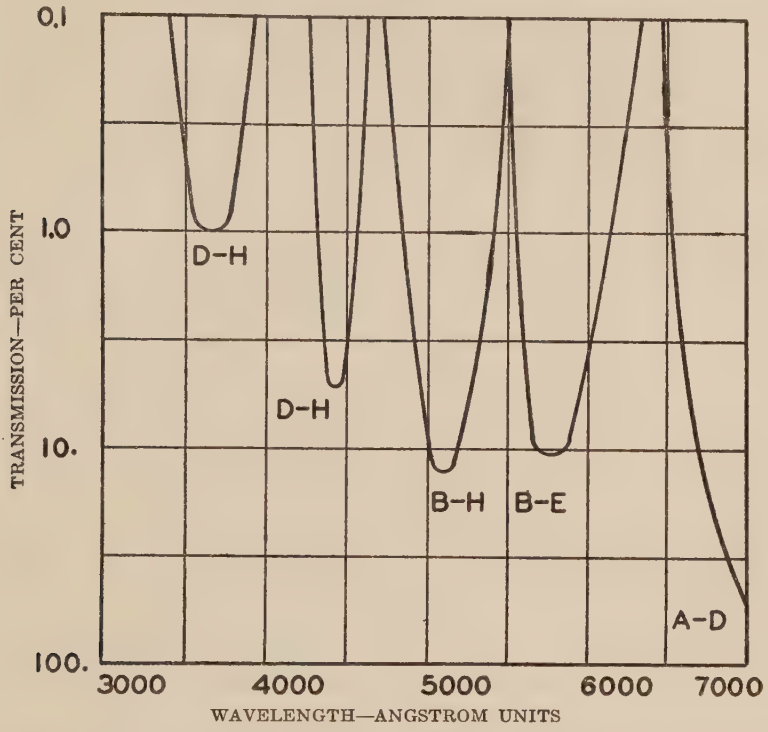
FIG. 2—*Response of Wild, Sepia, Vermilion, White and Brown at Various Wavelengths*



the light adjusted to give a reading of 10 foot-candles on the meter for each filter. After the flies were placed in the cell, readings were taken at 1-minute intervals. In each case, the lowest reading, corresponding to the maximum number of flies in the illuminated field, was recorded. Five readings were taken for each filter and the average calculated. Fig. 2 illustrates the curves obtained by this method.

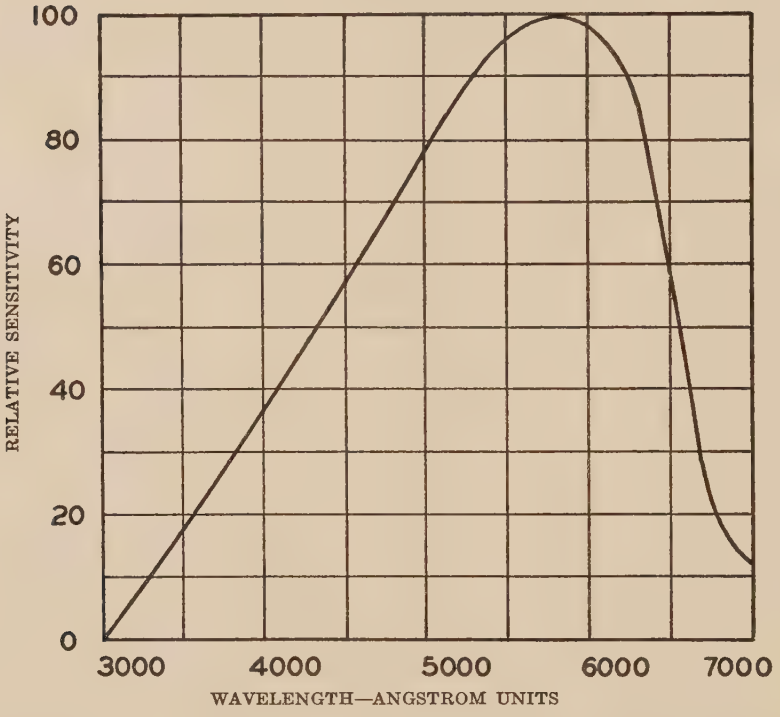
To obtain more accurate results it was soon found advisable to rearrange the apparatus and improve the method of taking readings. Each Wratten filter included quite a band of wave-

FIG. 3—*Transmission of Wratten "M" Filter Combinations*



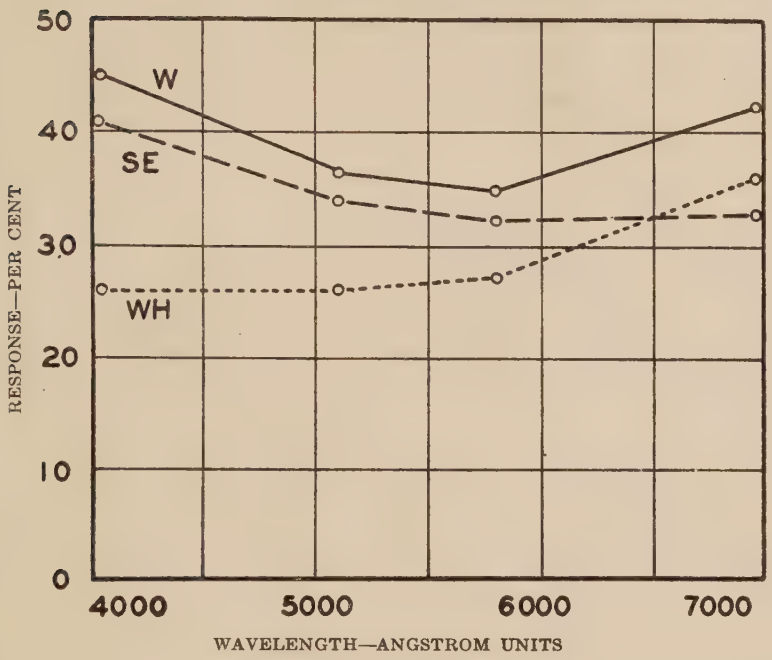
lengths and in many instances the filters overlapped considerably. Sharper bands were obtained by using the filters in pairs. The wave-length limits and transmission percentages are given in Fig. 3 as determined by the Eastman Kodak Company. Corrections were then made for the variation in spectral sensitivity of the Photronic cell. Fig. 4 shows the relative sensitivity of the cell for various wavelengths. The Photronic cell only was used in this set-up, the meter being replaced by a D'Arsonval galvanometer with a sensitivity of .00042 Ma/mm. The galvanometer settings for the different filter pairs were computed from the sensitivity curve of Fig. 4. The light intensity for the different filters was controlled by a slide-wire rheostat.

FIG. 4—*Spectral Sensitivity of Photronic Cell*



The results obtained by this method were quite different from the earlier experiments, as may readily be seen by comparing the curves of Figs. 2 and 5. Where formerly it was found that all flies gave a maximum response in the violet region, now they appeared to respond also in the red region, in which the white-eyed flies reached a maximum. Continued experimentation, however, eventually gave results which were more comparable with those of the earlier experiments, i.e., the flies no longer responded to the longer wavelengths but again gave evidence of maximum response in the violet region. In an effort to determine the factors responsible for the inconsistencies, the apparatus was modified so that 2 fly cells could

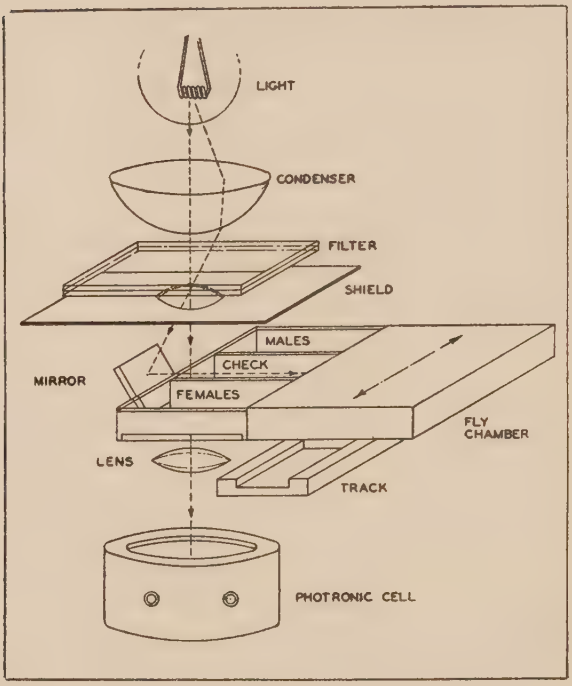
FIG. 5—*Response of Wild, White and Sepia at Various Wavelengths*



be placed alternately under the light, one containing females, the other, males. Though a difference was at first found to exist in the response activity between the two sexes, a complete reversal was soon manifested. At times the females showed greater activity, at other times, the males.

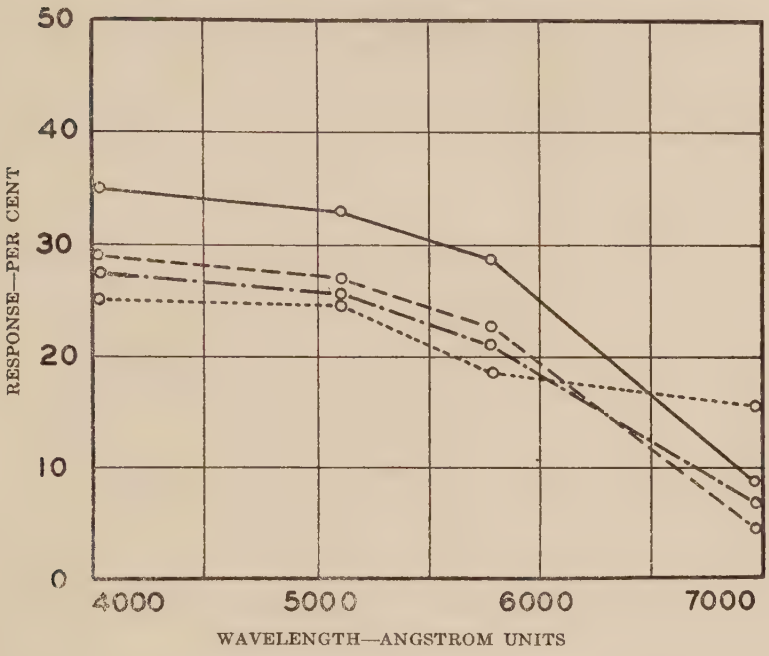
It was suggested that the relative position of the aperture, over the fly cell, to the light source was such that the flies could only be attracted to the light by the scattered illumination in the cell, i.e., if the flies were in the dark portion of the cell they could not see the light beam directly, and thus, depending upon the position they occupied at the time of observation, the response activity would vary correspondingly.

FIG. 6—*Response Measuring Device*



Another instrument (Fig. 6) was then designed and constructed, consisting of a light-tight box with the fly chamber divided into 3 compartments: one for males, one for females, and one empty compartment used for obtaining zero adjustment of the galvanometer. A 50-candle-power automobile lamp and a condensing lens were mounted on the outside of the box with provision for inserting color filters. As shown in the diagrammatic sketch, part of the light was reflected by a mirror into the fly chamber, and part transmitted through the chamber. With the aid of a lens, the images of the flies attracted by the light were projected upon the Photronic cell, which in turn was connected to the galvanometer. The fly chamber

FIG. 7—*Response of Sepia Males at Various Wavelengths*

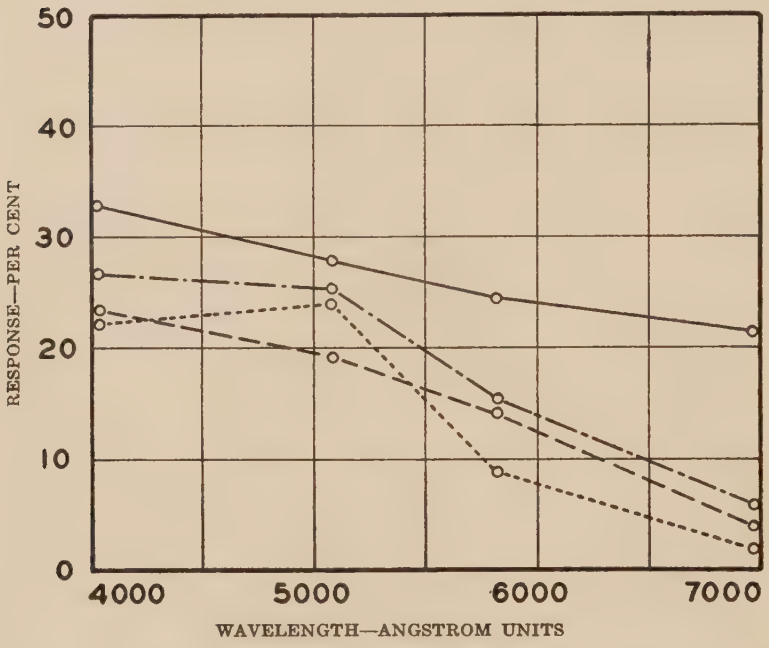


was mounted upon a track so that any one of the compartments could be placed in line with the light beam.

Corrections were made for the differences in body-size between the males and females. Twenty males and 20 females were killed by over-etherization and placed in their respective compartments of the chamber, and galvanometer readings taken. The difference in image area between the sexes was found to be 1 per cent.

In the experimental procedure, using the instrument described above, 50 adult flies of each sex were etherized and placed in the compartments. After the flies gave evidence of recuperation from the effects of the ether, a period of 15 min. was allowed before actual readings were begun. The box was closed, the light turned on, and zero adjustment made on the galvano-

FIG. 8—*Response of Sepia Females at Various Wavelengths*

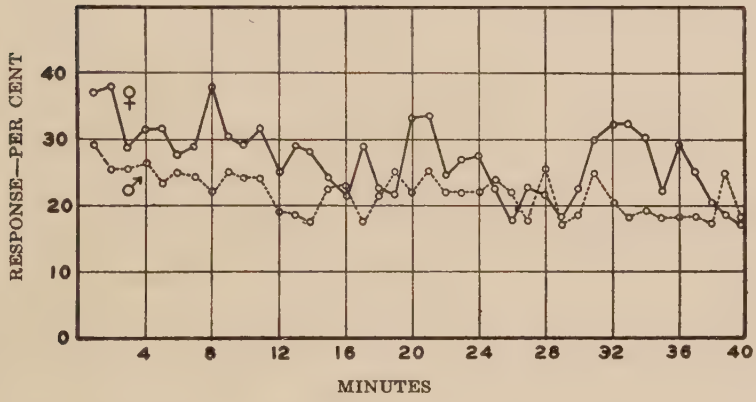


meter by placing the "check" compartment in position. No readings were taken until the flies had been in position under the light for 15 seconds, to allow for the galvanometer period. The lowest reading (corresponding to the maximum number of flies in the field) during a 1-minute interval was recorded. Five such readings were taken with each filter pair for each sex. These were expressed in percentage response and plotted against wavelength.

Sepia-eyed males and females were chosen for the first experiment with the instrument shown in Fig. 6. Again considerable variation was observed. The four separate experiments conducted with the flies are plotted in Figs. 7 and 8, which show the relative response of the males and females respectively. Though it is apparent that the maximum response

occurs in the 4000 Å. region for both sexes, there are nevertheless variations in the slopes of the curves and in the position occupied by male and female response. All other eye-color mutants available in the laboratory were again investigated and found to be as variable as before. The behavior of the sepia stock demonstrates the typical variations encountered.

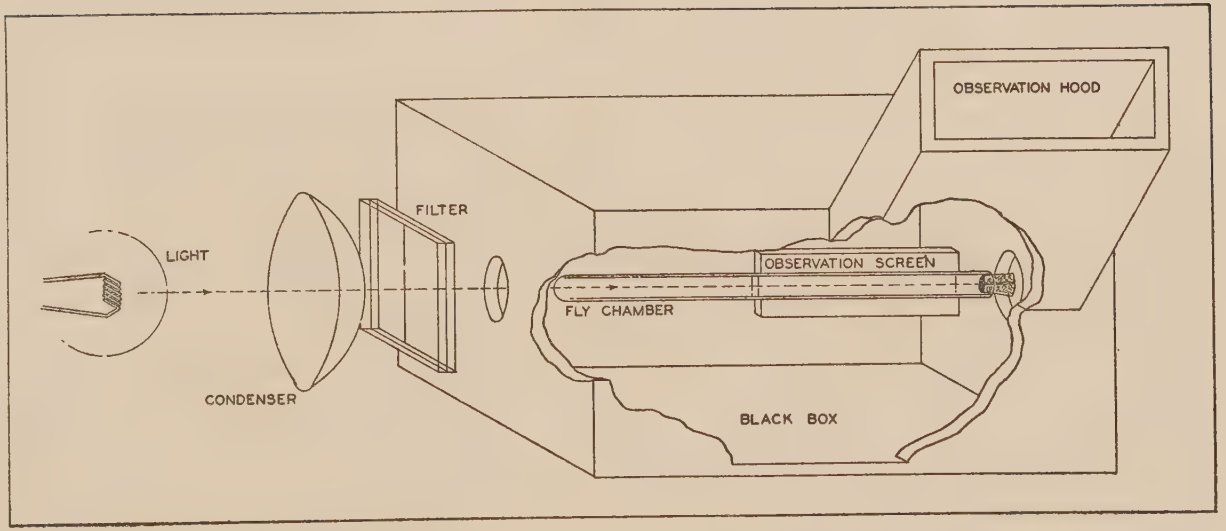
FIG. 9—*Fatigue of Wild-Type at* 4000 *Angstroms*



To determine whether the variations obtained during an experiment were due to fatigue, 50 males and 50 females were tested for response in the region of 4000 Å. A series of forty 1-minute intervals was taken with 1-minute rest periods. These experiments consumed the same length of time as was usually required for an experiment with the complete set of filters. The fatigue curves for the wild type *Drosophila* are given in Fig. 9. As can be observed from an inspection of the curves, though there are individual variations between readings and these variations are greater for the females than the males, there is some evidence of fatigue as indicated by the gradual decline.

Seeking further to explain the cause of the observed variations, the visual method was once more resorted to so that the

FIG. 10—*Counting Device*



This device was arranged for visual observation of the response of the flies. The fly chamber was inserted into a larger glass tube not shown in cut. The migration of the flies beyond the limits of the white observation screen was used as index of phototropic response.

behavior of the flies could actually be studied during the experiment.

A light-proof box of convenient size (Fig. 10) was prepared and through its center was inserted a large glass tube. This tube, which was to become the housing for the various fly chambers, had an observation screen attached to it. The fly chambers were constructed of glass tubing about 12 in. long and stoppered with a blackened cork. The source of light was supplied by a 50-candle-power automobile bulb housed in a brass tube which also contained a condensing lens. Between the brass lamphouse and the box proper was inserted a No. 47 Wratten blue filter. The filter was so mounted that a beam of light passing through it from the light source could readily pass through the end of the fly chamber.

The various types of flies were introduced into their respective fly chambers and the chambers inserted for an interval of 30 seconds into the large central glass tube. When the light was directed through the filter the blue light was perceived by the flies and they began to seek its source. A point, midway down the tube, at the end of the observation screen, was selected as the median point.

A group of stock culture bottles, containing respectively wild, vermilion, white, brown and sepia flies, were emptied of their contents of live flies. The eggs, larvae and pupae remained in the bottles. Sterile bottles containing media as recommended by Dr. C. B. Bridges were prepared. As the flies began to emerge in the old bottles they were transferred to the fresh culture bottles. These transfers were continued until 100 males and 100 females of each eye-color were secured. In the case of the white flies, there was tardiness in the emergence and consequently the reaction of these flies could not be studied in the first two experiments. After 24 hr. the transfers were discontinued, except in the case of the white

flies. Forty-eight hours were required to secure a sufficient number of these flies for the experiment.

The various types of flies were etherized and immediately separated according to sex. Fifty flies of each sex were placed in their proper fly chambers. Each chamber was marked with the type and sex of the flies contained therein, so that the various fly chambers might be kept separated. One-half hour was permitted to elapse, following the return to normal activity, before the flies were used for experimentation.

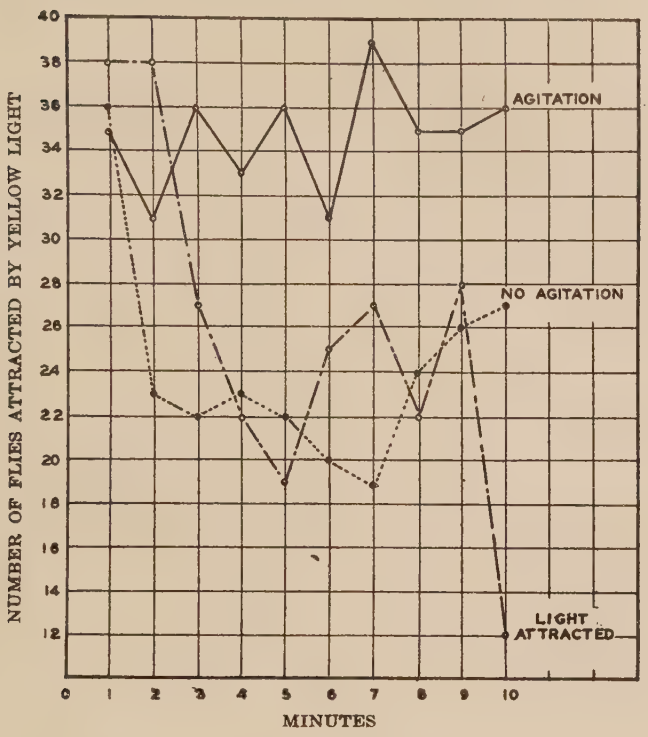
At the expiration of the ½-hour period the flies were subjected to the light for 30-second intervals. After a careful study of the travel of the flies, this interval was selected as that giving most dependable results. With shorter intervals the flies were not able to reach the median line, and with longer intervals many having passed the median line began to wander back. During the 30-second interval the number of flies passing the median line was recorded. This number was used as the index of the phototropic response of flies of this particular eye-color.

It was noted that many of the flies which did not reach the median line were occupied with the cleansing of their wings and bristles. Others, for reasons unaccountable, failed to show any activity whatsoever. It would seem, then, that these uncontrollable factors are at least in part responsible for the variations in our results.

It was observed also that the flies with different colored eyes proceeded toward the light at different rates. The sepia-eyed flies moved toward the light at a fast pace, while brown and wild moved rather slowly. Vermilion and white proceeded at a medium pace. Notwithstanding the varying speeds of the individual flies, the 30-second interval proved satisfactory for a response time-limit.

Since, in a series of preliminary experiments, great variations in counts were obtained, several methods of counting were investigated. In the first test, flies were permitted to

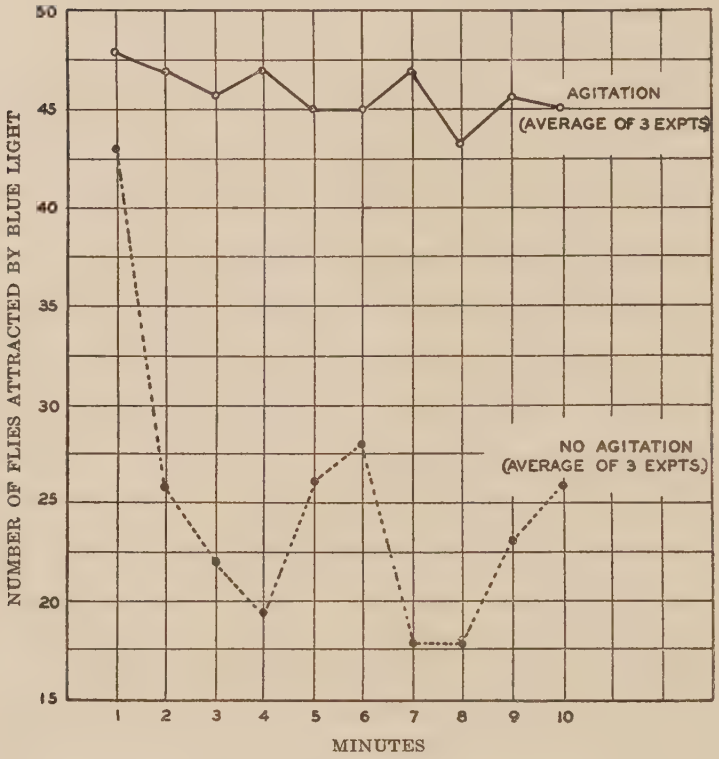
FIG. 11—*Response with and without Agitation*



diffuse throughout the length of the tube before their attraction to the light was studied. In another experiment the flies were drawn to the end of the tube by means of a strong white light before turning on the blue light at the other end. In still another experiment the flies were shaken to the end of the tube before each reading.

The results of these experiments are shown in Figs. 11 and 12. It is worthy of note that the least variation in response was found when the flies were brought back to the end of the tube by manual agitation. Greater variation was found when the flies were brought back to the end of the tube by means of the white light. It seemed that, having been subjected to the white light for a certain period, the flies were extremely

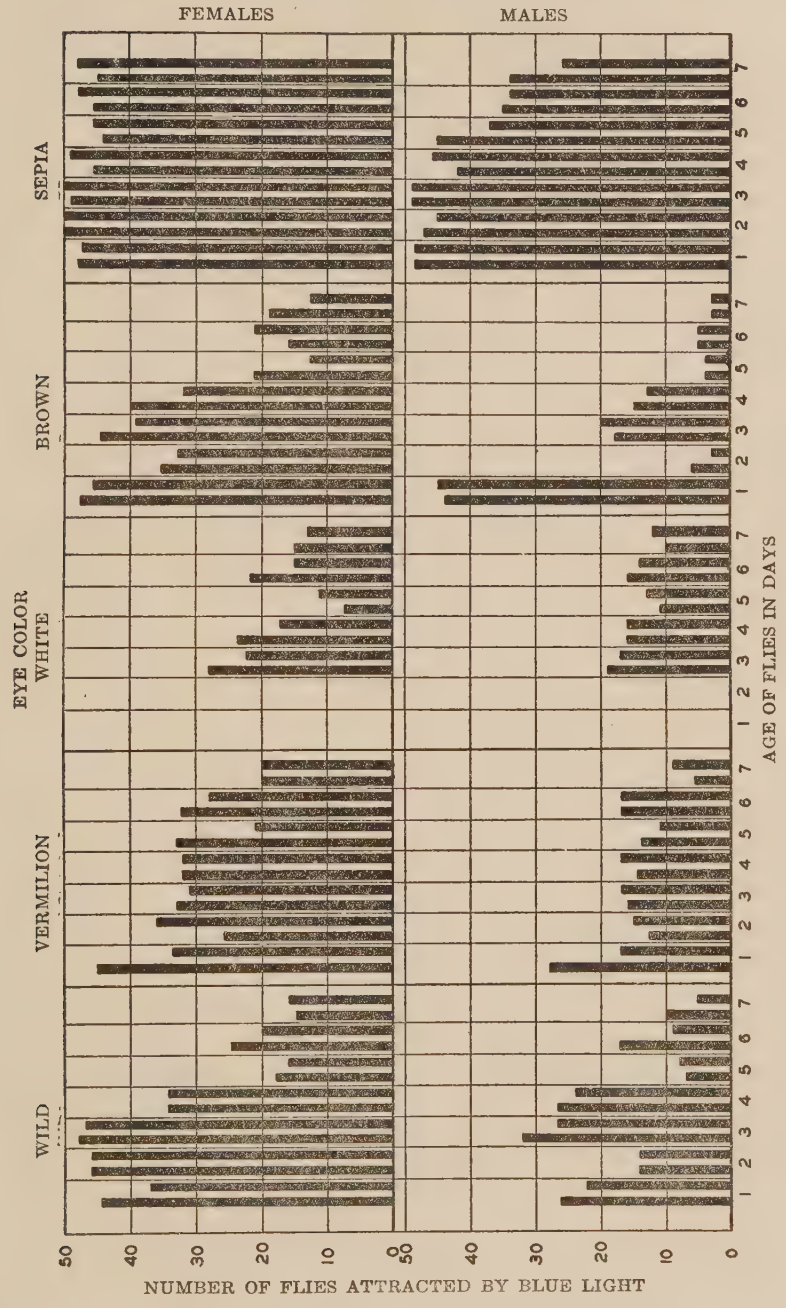
FIG. 12—*Response with and without Agitation*



slow in accommodating themselves to another stimulus, viz., the blue light. Where flies were permitted to diffuse throughout the length of the tube, the greatest variation in response was observed. Thus, these experiments established the necessity of agitation and the return of the flies to the end of the tube opposite the source of light before readings were begun.

The chart (Fig. 13) illustrates the results obtained for the various eye-colors over a 7-day period, using the agitation method. It shows that even in such a short period as 7 days, variations among the sexes and among the various mutants appear with irregular frequency.

Fig. 13—*Response of Various Mutant Eye-Colors of Drosophila Melanogaster to Blue Light*



It will be noted that the flies showing greatest and most consistent response were the sepia-eyed. The males did not vary in response more than a few per cent throughout the 7-day test period. The females, however, showed slight variation during the first 3 days, but during the next 4 days their response showed a decline. Among the brown males there was a gradual decline in response as the days progressed, but among the females there was a sharp decline following the initial reading. After attaining an extremely low point these brown females showed variations of only a few per cent. With white and vermilion there was, in general, but slight variation in the daily readings. The response of the males was greater than that of the females—a characteristic of all types of flies at this date. Next to brown, the wild-type presented greatest variation both within and between the sexes. Here, again, was observed a drop in response, but contrary to the other mutants, the wild-type dropped immediately after the initial readings to rise a day or so later to a new high. From this high point the gradual decline, with minor fluctuations, set in. These tests are to be continued throughout the life-span of the flies by the method of visual observation, as described above.

CONCLUSIONS

At the present time little significance can be attached to the variations in response, as shown in the chart of Fig. 13. However, it is obvious that males and females do not respond alike; also, there is some difference between the response of various eye-mutants. Only continued observation over an extensive period may reveal certain fundamental principles concerning phototrophism and its genetic control.

The apparatus shown in Fig. 6 will be modified so that the flies can be agitated before each reading interval. The experiments will be continued, using the entire series of filter pairs.

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McEwen, R. S., *J. Exp. Zool.*, **25** (1918): 49.

Payne, F., *Biol. Bull.*, **21** (1911): 297.

THREE METABOLIC STIMULATING FACTORS

By ROBERT J. NORRIS and CORNELIUS W. KREKE

In 1901[1] Wildiers found that the filtrate from boiled yeast cells contained a chemical substance affecting the growth and fermentation of yeast. He showed that this filtrate was soluble in water, 80 per cent ethyl alcohol, or 80 per cent ether, that it was fairly stable in the presence of acids and alkalis, was dialyzable and was not precipitated by lead acetate. While he observed that both the fermentation and the growth of the yeast were greatly stimulated by the addition of this filtrate, yet he did not differentiate between the factors stimulating growth and fermentation.

Euler and Petterson[2] found that the extracts which they obtained from various sources did not stimulate growth and fermentation in equal measures. They distinguished between the growth-promoting factor B I and the fermentation-promoting factor B II. By further boiling in acid solution they separated the fermentation factor B II into B II and B III.[3] B III was unstable and was considered identical with Harden's co-enzyme.

Fulmer and Nelson[4] fractionated the growth factor by extracting the ether extract with absolute alcohol. This gave them extract A, which was soluble both in water and absolute alcohol and left a solid, extract B, which was soluble in water only. The two together were much more potent than either alone.

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[1]Wildiers, E., *La Cellule*, **18** (1901): 313.

[2]Euler, H. von, and Petterson, A., *Z. physiol. Chem.*, **114** (1921): 4; *Expt. Sta. Record*, **46** (1922): 866.

[3]Euler, H. von, and Myrback, K., *Z. physiol. Chem.*, **115** (1921): 155; *Expt. Sta. Record*, **46** (1922): 866.

[4]Fulmer, E. I., and Nelson, V. E., *J. Biol. Chem.*, **51** (1922): 77.

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Miller[5] separated bios obtained from malt combings into two fractions by precipitation with barium sulphate; the solid he called Bios I and the filtrate Bios II.

Eddy, Kerr and Williams[6] succeeded in isolating these substances in pure crystalline form, whose theoretical formula was $C_5H_{11}NO_3$ and melting point 223° C.

Lucas[7] separated Bios I and Bios II by means of barium hydroxide precipitation and then purified Bios I by precipitating with lead acetate, and Bios II by dissolving in acetone. He found that the fraction of Eddy's stimulated only the strain of yeast used by Eddy, while his own factors stimulated both types of yeast.

Fulmer, Duecker and Nelson[8] fractionated an extract of ground alfalfa with 95 per cent alcohol, according to the method of Osborne and Wakeman.[9] They obtained four factors designated as No. I, II, III and IV. All four samples gave definite stimulations, but the only combination of optimum concentration which greatly improved the yields was a combination of I and III. They concluded that bios consisted of at least two fractions.

Eastcott[10] prepared Bios I and Bios II according to Lucas' method and obtained a pure extract of Bios I by neutralizing the lead acetate solution with sulphuric acid, heating with hydrochloric acid and precipitating Bios I in pure form with methyl alcohol. The compound had a formula of $C_6H_{12}O_6$,

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[5]Miller, W. L., *Science*, 59 (1924): 197.

[6]Eddy, W. H., Kerr, R. W., and Williams, R. R., *J. Am. Chem. Soc.*, 46 (1924): 2846.

[7]Lucas, G. H. W., *J. Phys. Chem.*, 28 (1924): 1180.

[8]Fulmer, E. I., Duecker, W. W., and Nelson, V. E., *J. Chem. Soc.*, 46 (1924): 723.

[9]Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 49 (1921): 63.

[10]Eastcott, E. V., *J. Phys. Chem.*, 32 (1928): 1094.

was found to be optically inactive, and was identified with inactive inositol. Eastcott found that the combination of inositol and Bios II greatly increased the growth of yeast and that Bios I or Bios II alone had no effect, but the two together possessed great activity.

Copping[11] showed that yeasts which did not respire required bios in order to grow in a synthetic mineral medium. A small amount of bios added to the yeast culture caused no immediate increase in respiration or fermentation, but after 24 hr. a slight increase in respiration and a great increase in fermentation were observed. Since our results show that, under proper conditions, bios immediately stimulates respiration, it is apparent that Copping's increase in respiration, which resulted only after a period of incubation, was due to an increase in the number of cells. She found that the primary effect of bios was to stimulate growth and that there was no parallel between growth, fermentation and respiration of yeast.

Narayanan[12] prepared a bios concentrate from an alcohol extract of yeast by hydrolyzing with barium hydroxide and autoclaving. The filtrate was freed of barium by means of sulphuric acid and the remaining filtrate treated with an excess of lead acetate. After the removal of lead 80 per cent alcohol was added to the filtrate and the active bios fraction was then precipitated by phosphotungstic acid. Still greater purification was obtained by precipitating the inactive impurities with silver. He found that his product promoted the growth of yeast and, through comparative tests with inositol, found that the latter did not promote the growth of yeast. His product resembled that of Wildiers but he disagreed with him as to the nature of this substance, Wildiers believing it to be an

[11]Copping, A. M., *Biochem. J.*, 23 (1929): 1050.

[12]Narayanan, B. T., *Biochem. J.*, 24 (1930): 6.

organic nitrogen base, and Narayanan a simple nitrogen compound.

Philipson[13] submitted the Z factor, or fermentation factor, to the procedure of Lucas for the separation of Bios I and Bios II. Like Bios II it was left in the filtrate, but unlike Bios II, it showed no growth activity, not even when inositol was added. A partial separation of the Z factor was accomplished by precipitating it with mercuric chloride and adding sodium hydroxide to pH 10.

The sum of the activities of the separate fractions was equal to the original solution, but the activity of the combined fractions was a little less than that of the original solution. Addition of inositol to fraction II gave the same result as fraction I added to fraction II. He concluded therefore that factor Z was not a single substance.

Williams and Bradway[14] concluded from their experiments that the bios extracts of various experimenters showed certain specificity to various brands of yeast, which may be some indication of the complex nature of these stimulating factors.

The work of Williams and Truesdail[15] seems to give further confirmation to this idea.

Miller, Eastcott and Sparling[16] prepared Bios I and Bios II by the method of Lucas and then, by neutralization with calcium hydroxide and treatment with charcoal and acetone, fractionated Bios II into Bios II A and Bios II B. In their tests each single factor gave a very small yeast crop as did also any two of them, and only when the medium contained all three fractions was the crop large.

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[13]Philipson, T., *Z. physiol. Chem.*, **193** (1930): 15.

[14]Williams, R. J., and Bradway, E. M., *J. Am. Chem. Soc.* **53** (1931): 783.

[15]Williams, R. J., and Truesdail, J. H., *J. Am. Chem. Soc.*, **53** (1931): 4171.

[16]Miller, W. L., Eastcott, E. V., and Sparling, E. N., *Trans. Roy. Soc. Can.*, III, **26** (1932): 165.

Euler and Larsson[17] concluded from their experiments that Lucas' method did not fractionate, claiming that Bios II contained practically all of the growth factor. Attempts to separate the fermentation factor Z from bios gave negative results, hence they decided that Bios II and the Z factor were the same.

Miller[18] found that small amounts of β-alanine added to a medium containing dextrose, salts, inositol and Bios II B, increased the yield of the yeast to a much greater extent than any other chemical which had been previously tried. The addition of *l*-leucine to media containing β-alanine caused further increase in the yeast crop.

It is evident at the present time that, although the arguments on the multiple sides of the question are numerous, the knowledge of the nature of this chemical substance is not at all certain. Very weighty arguments seem to come from the Toronto laboratories where Miller, Eastcott and their coworkers recognized bios to consist of inositol, Bios II A, Bios II B and Bios V. Euler and Larsson[19] seem to have shown definitely that factor Z and bios are one and the same; but they recognize only Bios II as the factor responsible for stimulation and give data to show that bios cannot be split into two fractions by Lucas' method, upon which Miller and Eastcott have based all their claims.

Little has been done to determine the relation between the factor stimulating respiration and the factors stimulating fermentation and growth. Norris and Ruddy,[20] working in these laboratories, showed that when yeast was irradiated with ultraviolet, a substance was liberated from the cells which

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[17]Euler, H. von, and Larsson, H., *Z. physiol. Chem.*, **223** (1934): 189.

[18]Miller, W. L., *Trans. Roy. Soc. Can.*, III, **30** (1936): 99.

[19]Euler, H. von, and Larsson, H., *loc. cit.*

[20]Norris, R. J., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 53.

greatly stimulated yeast respiration. This substance they found also in Marmite and a concentrate prepared according to Narayanan's[21] method. The stimulation of respiration did not parallel the stimulation of growth. Samples were taken at each step in the process of Narayanan and tested for their activity. In no case were the stimulations proportional.

The present experiments were undertaken to shed further light on the nature of the factors responsible for growth, respiration and fermentation and to determine if the stimulation of each biological process requires the presence of one or more of the factors.

EXPERIMENTAL

The source of the bios concentrates was malt combings.* Although the amount of bios in these combings is not very great, methods have been devised which permit an easy concentration of the active factor. The procedure of Lucas[22] was used and the samples were saved at each step of the process.

Preparations of the Bios Samples

One liter of water was heated to 85° C. and into this were stirred 150 g. of combings. After extracting for 15 min. the mixture was filtered, the heaviest residue being removed by filtering through cheese-cloth, the others, by centrifuging. Twenty-five cc. of the filtrate were removed and labeled *Sample 1.* The remainder of the filtrate, 738 cc., was evaporated *in vacuo* to one-fourth its volume. The temperature of the water bath was 90° C. and the vacuum was about 25 in. To the concentrated brew, 184 cc., was slowly added twice the amount of 95 per cent ethyl alcohol with constant stirring. A

*Obtained from the Bavarian Brewing Company, Inc., Covington, Kentucky.

[21]Narayanan, B. T., *loc. cit.*

[22]Lucas, G. H. W., *loc. cit.*

heavy gray precipitate, called *Sample 2*, was formed, which was filtered off through a Büchner funnel. The precipitate turned dark brown overnight. The filtrate had a deep red color and was acid to litmus. A portion of this filtrate was placed in a bottle labeled *Sample 3*. The remainder of the filtrate, 500 cc., was evaporated at 85° C. and 20 in. of vacuum, to one-tenth its volume. To this concentrate were added 24 cc. of a hot saturated solution of barium hydroxide and 72 cc. of 95 per cent alcohol. As the precipitation was incomplete, 108 cc. more of barium hydroxide and 220 cc. of 95 per cent alcohol were added to the mixture. The precipitate, which should contain the Bios I, was removed by filtration.

The filtrate, milky and yellow in color, was placed in a bottle and sterilized at 15 lb. pressure for 15 min. The filtrate was kept in this manner until the Bios I procedure was completed.

The Bios I precipitate was stirred four times with 92 cc. of water at room temperature and filtered each time. The combined volume of the washings was 369 cc. and was colored a deep red. The undissolved precipitate, which consisted of barium salts of protein decomposition products, was discarded. The solution was saturated with carbon dioxide and then boiled at 80° C. and 28 in. of vacuum to remove the excess carbon dioxide. The precipitate was filtered off and labeled *Sample 5*.

The filtrate was then heated to 70° C. and 15 cc. of a 2.5 normal solution of sulphuric acid slowly added. The precipitate was removed by filtering and labeled *Sample 6*. The filtrate was centrifuged clear of suspended matter and 25 cc. placed in a bottle and labeled *Sample 7*.

A new precipitate was formed in this filtrate by mixing with it 28.1 cc. of lead acetate containing 80 g. per liter and 6.8 cc. of normal ammonium hydroxide solution. The pre-

cipitate was separated by filtering and the filtrate labeled *Sample 8.*

The new precipitate was washed with 500 cc. of hot distilled water and then suspended in 100 cc. of the same fluid at room temperature and carbon dioxide passed through for 20 min. The mixture was filtered, the precipitate again suspended in 100 cc. of water, and carbon dioxide bubbled through for 15 min. The precipitate was again filtered out and the process repeated. The last precipitate was discarded because the quantity was so small that it was impossible to scrape it from the filter paper.

The combined filtrates were evaporated to one-twentieth their volume (36.9 cc.). A yellow precipitate formed, which was filtered off, washed with 25 cc. of water at room temperature and labeled *Sample 9.* To the 60 cc. of filtrate were added 240 cc. of 95 per cent alcohol. A white precipitate formed, which was removed by filtration. Twenty-five cc. of the filtrate were placed in a bottle labeled *Sample 10*, and the precipitate in a bottle labeled *Sample 11.*

The remaining 265 cc. of the filtrate were evaporated to 80 cc. at 65° C. and 25 in. of vacuum. Hydrogen sulphide was then bubbled through the concentrate for 15 min. The precipitated lead sulphide was filtered off and discarded. The filtrate was boiled *in vacuo* for 15 min. at a temperature of 85° C., 15 in. of vacuum, and then placed in a bottle labeled *Bios I Solution—Sample 12.*

The filtrate from the barium hydroxide-alcohol precipitation deposited a precipitate on standing. This was centrifuged. The clear filtrate again deposited a precipitate after standing for some time. Twenty-five cc. of this filtrate were set aside as *Sample 4.*

Carbon dioxide was passed through the remaining filtrate for one-half hour. Due to the precipitate already present it

was not possible to tell the nature of the new precipitate. The excess carbon dioxide was boiled off for 15 min. at 85° C. and a vacuum of 20 in. The precipitate was filtered out and labeled *Sample 13*. The filtrate was concentrated *in vacuo* to a volume of 125 cc., when the color had become a deep red. To this, 1400 cc. of acetone were added with constant shaking. At first a white precipitate formed, which disappeared on continued shaking. With further addition of acetone, an oily layer was formed which adhered to the sides of the vessel. This was filtered off, washed with acetone, dissolved in 95 per cent alcohol and placed in a bottle labeled *Sample 14*. The combined filtrate and washings were colorless. Twenty-five cc. of this filtrate were set aside as *Sample 15*, the remainder being evaporated at 85° C. and 20 in. of vacuum until the residue consisted of a gum and a red oily liquid.

This residue was extracted three times with 150 cc. of acetone. The acetone was warmed, shaken with the precipitate and then filtered. Twenty-five cc. of this filtrate were labeled *Sample 16*. The remaining precipitate was extracted with 95 per cent alcohol, whereupon the red oily liquid dissolved. This solution was called *Sample 17*. The gummy precipitate was dissolved in water and called *Sample 18*. The acetone filtrate was evaporated to 30 cc. at 50° C. and 28 in. of vacuum, when a thick, cloudy, reddish liquid was obtained. This was *Sample 19*.

Each of the nineteen samples was placed in a small evaporating dish and evaporated to dryness in an electric oven at 80° C. The remaining solids were carefully weighed and the solid content per cc. of each sample, as well as the total amount in each filtrate at every stage of the process of purification, was determined.

The results are shown in Table I.

KEY TO SAMPLE NUMBERS

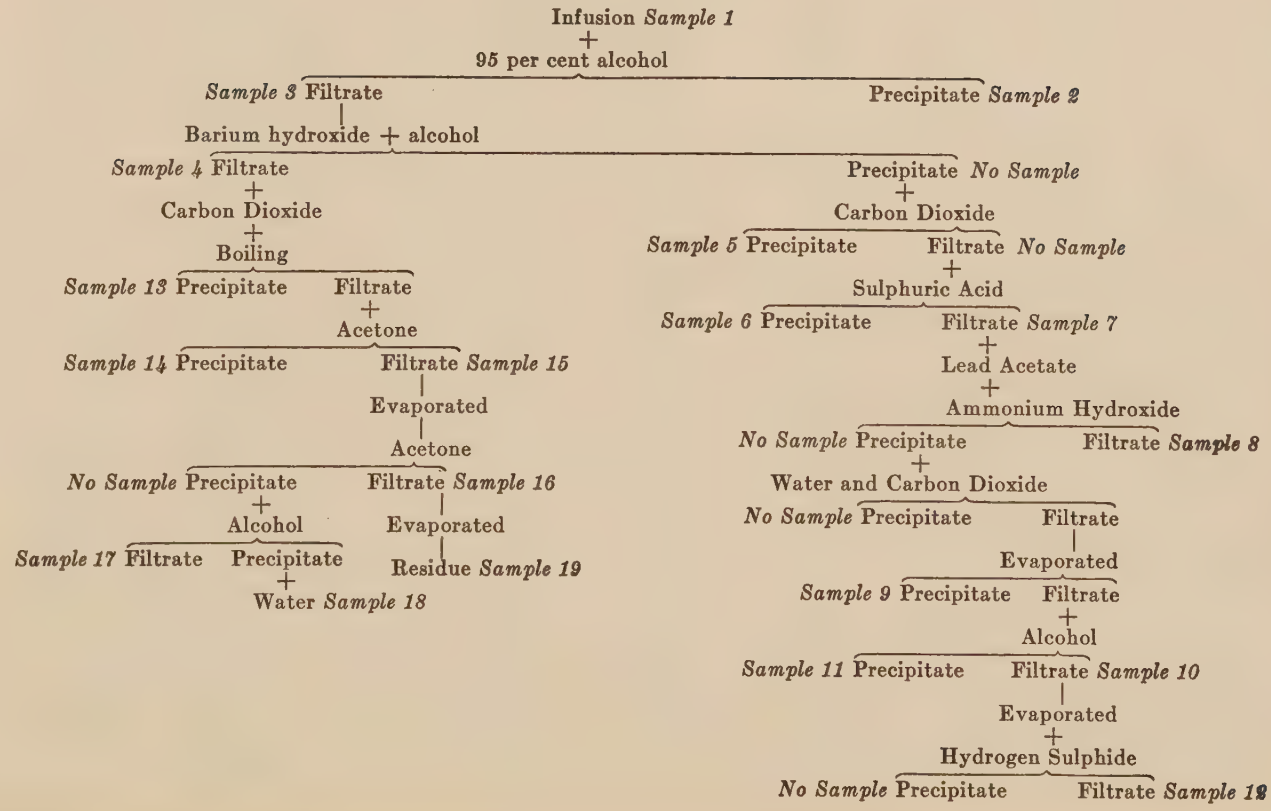

TABLE I

Solid Content of the Samples

	Number of Grams of Solid Content of Sample	Total Volume of Each Filtrate	Number of Grams of Solid Content in Total Filtrate
Sample 1	.....	763 cc.	.....
" 2	2.4444	.....	.....
" 3	1.2683	577 cc.	29.1709
" 4	0.6251	474 cc.	11.8769
" 5	.....	.....	.....
" 6	.....	.....	.....
" 7	0.2091	386 cc.	3.1365
" 8	3.0103	421 cc.	3.0103
" 9	.....	.....	.....
" 10	0.0256	.....	.....
" 11	.....	.....	.....
" 12	0.0944	30 cc.	0.0944
" 13	.....	.....	.....
" 14	6.4268	.....	6.4268
" 15	0.0947	1600 cc.	6.1008
" 16	0.1059	450 cc.	1.9062
" 17	0.8188	100 cc.	0.8188
" 18	1.6835	100 cc.	1.6835
" 19	1.3257	30 cc.	1.3257

The samples were redissolved in distilled water, the concentrations usually being 50 mg. per cc. In some cases, however, the amount of solid did not permit so high a concentration. The samples were sterilized at 15 lb. pressure and then adjusted to pH 7.3 by the colorimetric method with one-fiftieth normal sodium hydroxide.

Growth Technic

The yeast used in testing the growth activity of the different samples was obtained in pure sterile condition by plating *Saccharomyces cerevisiae* on Sabouraud's medium and transferring from day to day to Sabouraud's slants. The yeast suspension was prepared by washing the 48-hour growth of five slants into 100 cc. of sterile Reader's medium.[23] Each bios sample, when prepared for testing its growth activity, was diluted with Reader's medium to the various concentrations to be used.

Each rocker tube contained 1 cc. of the concentration of the sample and enough yeast suspension so that when it was diluted to 25 cc. with Reader's medium, the yeast count was 1. The rocker tubes were rocked 60 times per min. for 24 hr. in a water bath maintained at a constant temperature of 30° C. and thereafter the yeast growth determined. The yeast count was determined in all cases by a photodensitometer,[24] which measured the number of cells by the amount of light absorbed. This instrument was standardized by hemacytometer count.

The results of the growth activity of the different samples are given in Table II and are expressed in growth units per milligram. A growth unit is considered that concentration of bios expressed in milligrams per cubic centimeter of solid content which would give a count of 25 over that of the control.

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[23]Reader, V., *Biochem. J.*, **21** (1927): 901.

[24]Beck, W. A., *Science*, **85** (1937): 2206.

TABLE II

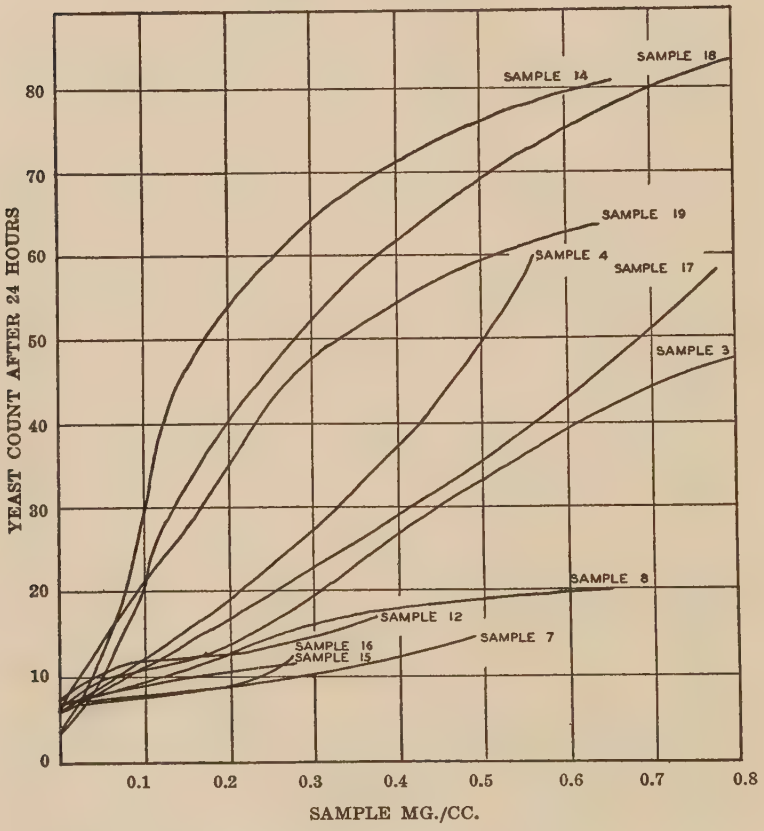
Comparison of the Growth, Respiration and Fermentation Activity of the Various Samples of Bios

	Growth units/mg.	Respiration units/mg.	Fermentation units/mg.
Sample 3	2.08	1.13	2.32
" 4	4.13	1.07	2.85
" 7	0.615	Slight stimulation	.....
" 8	0.38	1.04	0.909
" 12	0.65	1.22	.....
" 14	10.60	1.5	0.377
" 15	1.04	No stimulation	.....
" 16	1.00	0.66	.....
" 17	2.29	1.33	1.47
" 18	6.28	3.33	1.0
" 19	5.88	1.4	0.625

To insure identical conditions under which these units were determined, the synthetic medium should be Reader's, the tubes seeded with a count of 1, and rocked 60 times per min. for 24 hr. at a temperature of 30° C.

Stimulation curves of the various samples are given in Fig. 1.

FIG. 1—*Growth Activity of Various Samples*



Respiration Technic

The respiration activity of the different samples was determined by the "direct method" of Warburg.[25] The yeast suspension was prepared by shaking 3.5 g. of Fleischmann baker's yeast* with 200 cc. of Ringer phosphate glucose solution which had the following composition per 1000 cc. of solution:

Sodium chloride	0.900 g.
Calcium chloride	0.242 g.
Potassium chloride	0.011 g.
Glucose	0.200 g.
Sodium dibasic phosphate	0.059 g.
Sodium acid phosphate	2.05 g.

This suspension was measured on the photodensitometer and diluted with more Ringer solution until a count of 250 was obtained. This yeast count was decided upon as a result of preliminary tests.

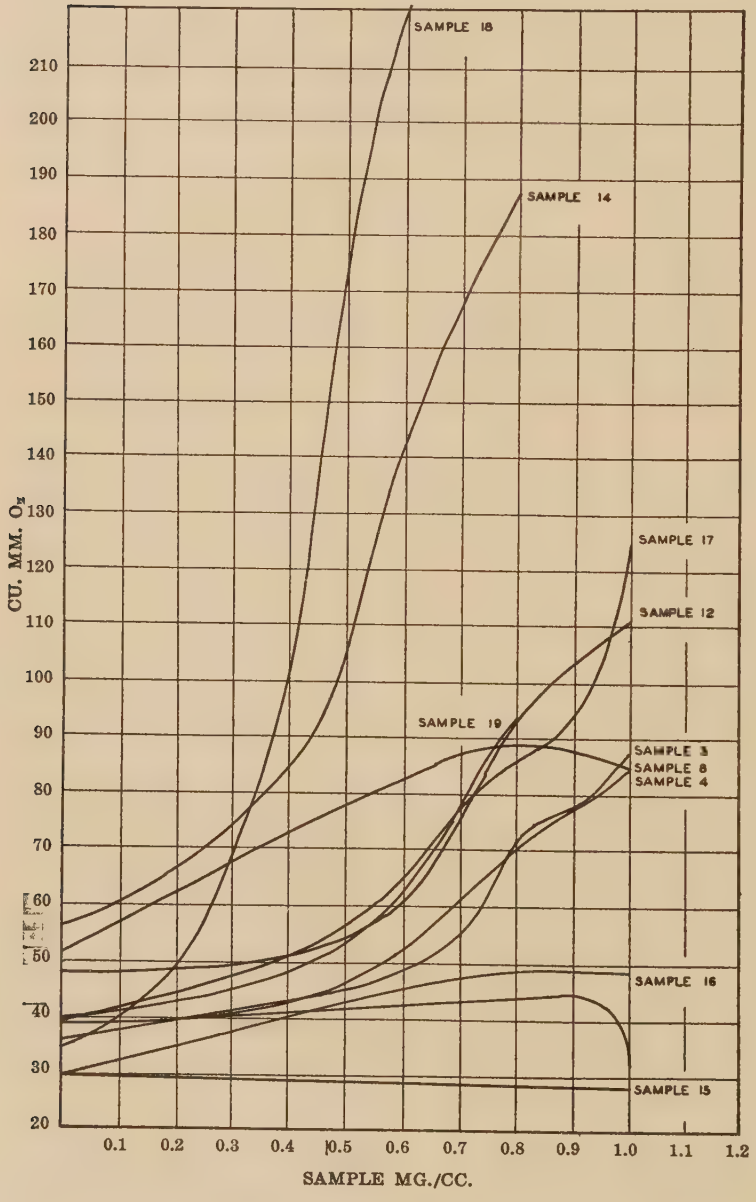
The bios samples were diluted to the various concentrations to be tested with Ringer solution. Two-tenths cc. of normal potassium hydroxide solution was placed in the inner well of each manometer flask. The outer well of each flask contained 1 cc. of a bios concentrate, 1 cc. of yeast suspension and 1.1 cc. of Ringer phosphate solution, so that in all cases the total volume of the different constituents was 3.3 cc.

In the control flask the 1 cc. of the bios concentrate was replaced with 1 cc. of Ringer solution. A barometric control was also run with each experiment to correct for changes

*It is not necessary to use an absolutely sterile strain of yeast because the slight contamination by bacteria or mould would not influence the oxygen-uptake to any appreciable extent in the short time required for the measurement.

[25] Warburg, O., *Z. physiol. Chem.*, 92 (1914): 231; *Biochem. Z.*, 142 (1923): 317; and 152 (1924): 51; Warburg and Kubowitz, F., *Biochem. Z.*, 214 (1929): 7; Warburg, Kubowitz, F., and Christian, W., *Biochem. Z.*, 242 (1931): 170.

FIG. 2—*Respiration Activity of Various Samples*



in barometric pressure. This flask contained 0.2 cc. of the hydroxide solution and 3.1 cc. Ringer solution.

In testing the respiration activity of the different samples the manometers were shaken 115 times per min. in a water bath at a constant temperature of 37.5° C. for an equilibrium period of 15 min., during which time the Brodie solution[26] in both tubes was open to the air. This period allowed the solutions and air in the flasks to come to the temperature of the water bath. The one tube was then so closed that it communicated only with the flask. Shaking was continued for a respiration period of 25 min.

The water bath, which was maintained at a temperature of 37.5° C., was enclosed in a box whose temperature was approximately 27° C. The temperature of the box did not vary more than two degrees at any time. Since the respirometers had previously been adjusted to a temperature of 37.5° C., which was optimum for animal tissues, it was necessary to run yeast respiration at this temperature rather than at the optimum temperature for yeast, namely, 30° C.

The results on yeast respiration are given in Table II and are expressed in respiration units per milligram; one respiration unit being that amount of bios in milligrams per cubic centimeter which is capable of stimulating the respiration of the test sample 100 per cent over that of the control in a respiration period of 25 min.

A comparison of the stimulation of the various samples is shown in Fig. 2.

Fermentation Technic

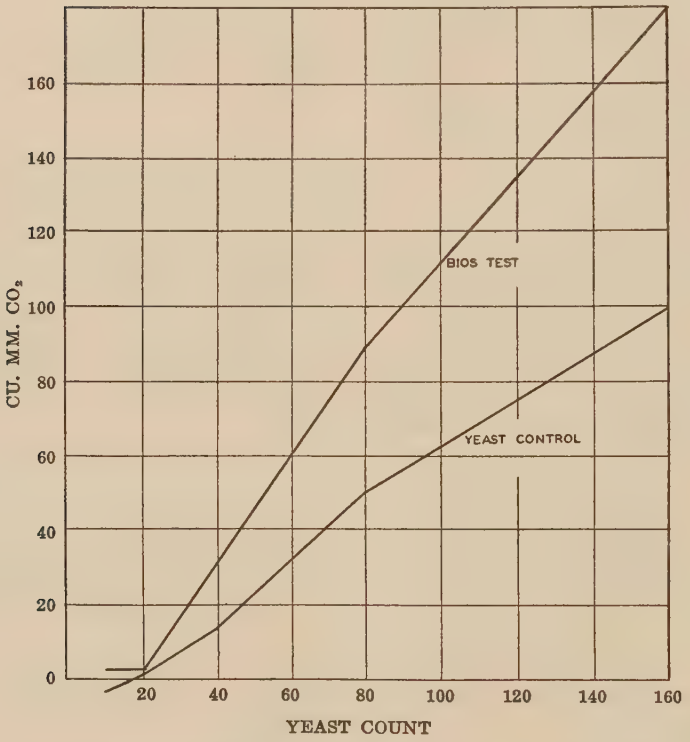
The fermentation activity of the different samples was determined by the "direct method" of Warburg. It has been shown by Slator[27] that the fermentation is directly proportional

[26]Dixon, M., Manometric Methods (1934).

[27]Slator, A., *J. Chem. Soc.*, 89 (1906): 128; 93 (1908): 217.

to the number of yeast cells only when the fermentation is independent of the amount of fermentation medium present. Slator showed that in the case of glucose, if the concentration was 3 or 4 per cent or greater, the fermentation was indepen-

FIG. 3—*Fermentation Activity of Sample 14 as Function of Yeast Count*



dent of the amount of glucose present. In our experiments the Ringer phosphate glucose solution was so fortified with a 12 per cent solution of glucose that the total constituents in the outer well of the manometer flasks, whose volume was 3.1 cc., had a glucose concentration of approximately 4 per cent. Into the inner well 0.2 cc. of ordinary Ringer solution

was placed to compensate for the hydroxide of the respiration experiments. The outer well contained, besides the 1.1 cc. of the fortified Ringer solution, 1 cc. of yeast suspension and 1 cc. of a bios concentrate.

In the control flasks the 1 cc. of the bios sample was replaced by 1 cc. of ordinary Ringer solution and in the barometric control the yeast and bios were replaced with Ringer solution.

The yeast suspension was prepared by shaking 1 g. of Fleischmann baker's yeast in 200 cc. of Ringer solution. This suspension was measured on the photodensitometer and diluted with more Ringer solution until the count was 80. This count was used because it was found from the results shown in Fig. 3 that for this and greater concentrations the stimulation is proportional to the yeast count. Below 80 the stimulation is variable.

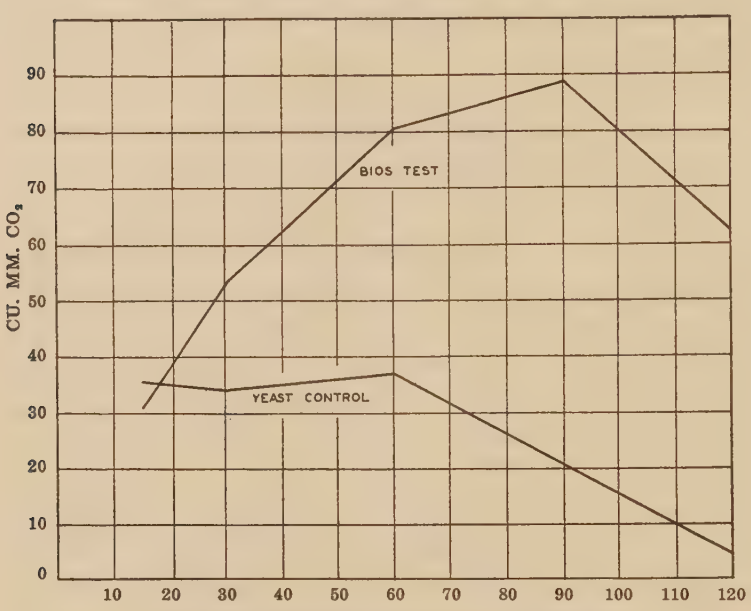
The bios sample used to determine this optimum yeast count was *Sample 14* (acetone precipitate) diluted to 25 mg. per cc. One cc. of this dilution was used in these tests.

At the beginning of each experiment all the solutions in the manometer flasks were saturated with carbon dioxide by bubbling the gas through them for 5 min. before placing them in the flask. After the flasks were fastened to the manometers, carbon dioxide was passed through each manometer for 2 min. to insure complete exclusion of the air. This flushing and saturating with carbon dioxide was to insure immediate reading of the carbon dioxide production and the prevention of any respiration of yeast due to the presence of oxygen. The manometers were then placed in the water bath at the usual temperature, 37.5° C., and shaken with the stopcocks open for 1 hr. as an equilibrium period, and then 5 min. with the stopcocks closed to measure the fermentation.

The reason for the 1-hour equilibrium period is at once apparent from an examination of Fig. 4. This graph is very

FIG. 4—*Fermentation Activity of Sample 14 as Function of Time*

(8 MG./CC.)

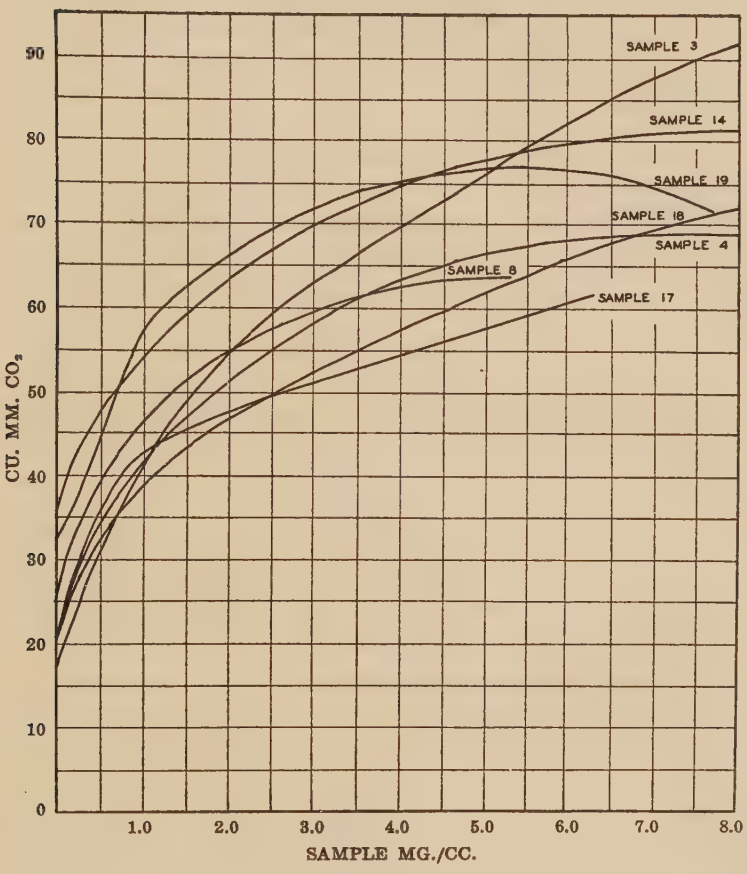


interesting from the standpoint of the mechanism of the action of bios. At first there is an inhibitory effect which disappears after some 18 min., when stimulation begins. This stimulation gradually increases for 90 min., when it drops very sharply. The fermentation of the controls remains constant for about 1 hr., at which time it begins to drop. This mechanism is under investigation at the present time in our laboratories.

The results of these fermentation experiments are given in Table II, and are expressed in fermentation units per milligram. One unit is that amount of bios in milligrams per cubic centimeter which will give 100 per cent stimulation over the control when the equilibrium period is 1 hr. and the fermentation period 5 min. and all other conditions as in these experiments.

The curves of Fig. 5 compare the carbon dioxide production of the different samples of bios at various concentrations.

FIG. 5—*Fermentation Activity of Various Samples*



It seems that practically all the growth activity is in the Bios II fractions, of which *Sample 14* (acetone precipitate) contains the most. *Sample 19*, which is the Bios II of Lucas, contains about one-half as many growth units per milligram as *Sample 14*. These results are all in accordance with the findings of Euler and Larsson.[28]

[28]Euler, H. von, and Larsson, H., *loc. cit.*

The respiration activity seems to be concentrated in *Sample 18*, which is the precipitate from the acetone extraction, soluble in water, but not in alcohol.

No purification of the fermentation factor seems to have been accomplished. *Sample 3*, the alcohol filtrate, and *Sample 4*, the filtrate from the barium hydroxide-alcohol precipitation, show the greatest potency.

In general, there appears to be no parallel between the stimulation of growth, respiration and fermentation. This indicates one of two things: either there is a specific factor (or factors) required for each process; or, there is one specific factor for each, and one common factor which supplements each of the specific factors.

Table III shows the total units of active materials in the filtrates and precipitates obtained in the various steps in the process of purification of bios, of which each sample is only a part.

As can be seen from this table, *Sample 14* is the best source for the growth factor, while *Sample 3* contains the fermentation factor in the most concentrated form. The respiration factor is in its most concentrated form in *Sample 18*, while the greatest quantity is found in *Sample 3*.

In order to show whether or not bios was split into Bios I and Bios II by the method of Lucas, the following experiment was performed. *Sample 7*, the filtrate before purification with lead acetate and supposed to contain Bios I in a somewhat impure form, was taken as representative of Bios I. *Sample 19* was used as Bios II. The rocker tubes were filled as follows:

Tube No. 1 contained 0.3 growth unit from *Sample 7* plus 1 growth unit from *Sample 19*.
Tube No. 2 contained 0.3 growth unit from *Sample 7*.
Tube No. 3 contained 1.0 growth unit from *Sample 19*.
Tube No. 4 contained the control.

TABLE III

Comparison of the Total Number of Growth, Respiration and Fermentation Units in each Sample of Bios

	Weight in Grams	Growth Units	Respiration Units	Fermentation Units
Sample 3.....	29.1709	60675.5	32963.1	67676.5
" 4.....	11.8769	49061.6	12708.3	25297.16
" 7.....	3.1365	1928.94	Very few	.....
" 8.....	3.0103	1143.9	3130.7	2736.4
" 12.....	0.0944	61.36	115.2	.....
" 14.....	6.4268	68124.0	9640.2	2422.9
" 15.....	6.1008	6344.8	None	.....
" 16.....	1.9062	1906.2	1258.1	.....
" 17.....	0.8188	1875.05	1089.0	1203.6
" 18.....	1.6835	10572.4	5606.0	1683.5
" 19.....	1.3257	7795.1	1855.9	828.56

Each tube was seeded with enough yeast suspension to give a count of 1 when the volume was made 25 cc. by the addition of Reader's medium. The tubes were shaken for 24 hr. at 30° C.

The results are given in Table IV.

TABLE IV

Comparison of the Growth Activity of Bios I and Bios II of Lucas

Tube	Transmission Percentage	Cell Count
No. 1	32.6	44.54
" 2	66.0	17.00
" 3	46.8	29.44
" 4	84.2	7.15

The combined effect of *Sample 7* and *Sample 19* is nothing more than additive. There appears to be no supplementary action. This is in accordance with the work of Euler and Larsson[29] who also found that bios was not fractionated by Lucas' method.

It was the intention of the authors to check definitely whether the Z factor of fermentation and the respiration factor were separated into two or more fractions, but, due to the very small amount of material remaining, the work had to be postponed until more samples would be available.

From the fact that *Sample 3* and *Sample 4* of Table II show approximately the same amount of fermentation and respiration units per milligram, it might be concluded that these factors are not fractionated and that the barium hydroxide-alcohol procedure is very inefficient in purifying the two factors.

[29]Euler, H. von, and Larsson, H., *loc. cit.*

SUMMARY

The factors stimulating growth, fermentation and respiration are not one and the same substance. The growth factor is concentrated in *Sample 14*, the respiration factor in *Sample 18*, and the fermentation factor in *Sample 3* and *Sample 4*.

The growth activity is wholly dependent on Bios II of Lucas and there is no separation into Bios I and Bios II by the barium hydroxide-alcohol precipitation. That the factors of fermentation and respiration are not fractionated by this precipitation may be concluded from Table II in which it is shown that no greater concentration is obtained.

Norris and Hart[30] showed that the stimulative factors cannot be separated by dialysis. This does not necessarily indicate that they are one and the same substance, but only that the size of the molecules must be approximately the same.

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[30]Norris, R. J., and Hart, Sr. M. Jane, O.P., *this publication*, **1** (1937): 65.

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Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, **49** (1921): 63.

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Reader, V., *Biochem. J.*, **21** (1927): 901.

Slator, A., *J. Chem. Soc.*, **89** (1906): 128; **93** (1908): 217.

Warburg, O., *Z. physiol. Chem.*, **92** (1914): 231; *Biochem. Z.*, **142** (1923): 317; and **152** (1924): 51.

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PROLIFERATION-PROMOTING FACTORS FROM ULTRA-VIOLET INJURED CELLS*

By GEORGE SPERI SPERTI, JOHN R. LOOFBOUROW and SISTER CECELIA MARIE DWYER, S.C.

In addition to the lethal action of ultra-violet radiation on microorganisms, long since well established, a cell-proliferation-promoting action has been noted by various workers under particular circumstances. Other investigators, endeavoring to demonstrate such an effect, have failed to find it. Contradictory evidence has likewise been presented regarding the ability of ultra-violet radiation to increase the rate of fermentation or respiration of microorganisms. Recently, explanations of the discrepancies have been advanced by workers in this laboratory,[1] and modifications in the technique of conducting such experiments have been described, which have led uniformly to positive results.

Briefly, the explanation of the stimulative actions advanced in these recent papers, is that cells injured by ultra-violet radiation or other means produce substances which promote the proliferation, fermentation and respiration of normal cells. The negative results so frequently obtained by various workers in tests of "stimulation" made on suspensions of cells directly irradiated, are explained in these papers as due either to the injury or killing being carried so far that the stimulative action is masked by the lack of sufficient uninjured, normal cells to exhibit the effect, or to the irradiation being insufficient to produce appreciable stimulating materials.

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*Research conducted under the Sir Charles F. Williams Fellowship.

[1]Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sr. M. Veronita, O.P., *Nature*, **139** (1937): 589; Fardon, J. C., Carroll, Sr. M. Jordan, O.P., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 17; Fardon, J. C., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 41.

REVIEW OF THE LITERATURE

Among the early evidence of the proliferation-promoting power of ultra-violet for microorganisms was the occurrence of larger colonies at the edges of irradiated areas on petri dish cultures. In our own experiments, ten years or more past, Sperti, Burger and Norris observed such effects during the irradiation of various kinds of bacteria and Loofbourow obtained similar results with yeasts and moulds. These observations were never published. They nevertheless excited our interest in the question of growth or proliferation stimulation, and paved the way for work which led to the present investigations.

Among others who have noted such results in carefully controlled experiments may be mentioned Browning and Russ,[2] Coblentz and Fulton[3] and Gates.[4] Browning and Russ used glass plates coated with agar and smeared with *Staphylococcus aureus*, which they irradiated in a quartz spectrograph, using a tungsten spark source. Growth was stimulated at the edges of the lethal spectrum area. Coblentz and Fulton, and Gates observed increased growth of survivors on plates irradiated until most of the cells had been killed. This they explained as due to the reduction in the number of colonies, permitting each colony to make use of a greater amount of nutrient material and reducing the accumulation of toxic waste products. Our interpretation would be that stimulating materials produced by the injured cells accounted (at least in part) for the results. Nadson and Philippov[5] irradiated petri dish smears of various yeasts and moulds, shielding outer portions of the plates.

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[2]Browning, C. H., and Russ, S., *Proc. Roy. Soc.* (*London*), *B* **90** (1917): 33.

[3]Coblentz, W. W., and Fulton, H. R., *Scientific Papers of the Bureau of Standards*, **19** (1924): 641.

[4]Gates, F. L., *J. Gen. Physiol.*, **13** (1929): 231.

[5]Nadson, G., and Philippov, G., *Compt. rend. soc. biol.*, **98** (1928): 366.

Increased development occurred near the borders of the irradiated areas.

Considerable evidence for the stimulation of microorganisms by ultra-violet has resulted from a study of the effects of these radiations on yeast. Among those who have found yeast growth to be stimulated by ultra-violet may be mentioned Frank,[6] Konarski,[7] Reinhard,[8] de Fazi[9] and Hinrichs.[10] Recently Hollaender and Duggar[11] found that whenever yeast was irradiated with ultra-violet of 2650 Å until about 10 per cent of the organisms survived, the survivors proliferated when transferred to salt solutions in which controls died.

Several investigators have found ultra-violet to promote the fermentation of yeast; for example, de Fazi and de Fazi.[12] Feuer and Tanner[13] pointed out quite plausible criticisms of the investigations of the de Fazis. Yet, other workers, as Gronchi,[14] Owen and Mobley,[15] and Owen,[16] have reported similar results. Lindner[17] observed even greater stimulation of fermentation than that reported by the de Fazis, but he found that irradiation in shallow layers resulted only in lethal action. His results seem reasonable to us in view of our explana-

[6]Frank, G., *Biol. Zentr.*, **52** (1932): 1.

[7]Konarski, J., *Acta Biol. Exptl. (Warsaw)*, **1** (1932): 276.

[8]Reinhard, A. W., *Compt. rend. soc. biol.*, **89** (1923): 1080.

[9]de Fazi, R., *Atti accad. Lincei*, VI, **5** (1927): 901.

[10]Hinrichs, M. A., *Proc. Soc. Exptl. Biol. Med.*, **26** (1928): 175.

[11]Hollaender, A., and Duggar, B. M., *J. Bact.*, **33** (1937): 16.

[12]de Fazi, R., and de Fazi, R., *Ann. chim. applicata*, **8** (1917): 93; *Atti accad. Lincei*, **31** (1922): 31; *Giorn. chim. ind. applicata*, **4** (1922): 463; *Ann. chim. applicata*, **6** (1917): 221; *Atti congr. naz. chim. ind.*, (1924): 449; *J. Ind. Eng. Chem.*, **13** (1921): 265.

[13]Feuer, B., and Tanner, F. W., *J. Ind. Eng. Chem.*, **13** (1921): 265.

[14]Gronchi, V., Nota I, *Boll. soc. ital. biol. sper.*, **7** (1932): 957.

[15]Owen, W. L., and Mobley, R. L., *Zentr. Parasitenk.*, II Abt., **88** (1933): 273.

[16]Owen, W. L., *Food Industries*, **5** (1933): 252.

[17]Lindner, P., *Wochschr. Brau.*, **39** (1922): 166.

tion of the effects, for irradiation in shallow layers might result in killing all of the cells under conditions in which only the surface layers of deep suspensions would be killed, leaving the other cells to cause fermentation under the stimulative action of the products of the injured cell.

Among those who have found only lethal effects, without stimulation, in the action of ultra-violet on yeast are Feuer and Tanner,[18] Schreiber,[19] Tanner and Ryder,[20] Tanner and Byerly,[21] and Söhngen and Coolhaas.[22] Abderhalden[23] noted the fermentation of yeast to be inhibited by ultra-violet or by the addition of irradiated fat extracted from yeast. In our opinion, the explanation of most of these negative results is that such conditions of irradiation were employed that stimulation was either masked by the killing of too many cells or failed to appear because insufficient cells were injured to produce a high enough concentration of stimulating materials. With regard to respiration, Norris and Ruddy[24] found that the presence of sugar in the yeast suspension caused such a great stimulation that any increase due to the stimulating action of the irradiation was totally masked.

Some investigators, notably Gronchi,[25] Guerrini,[26] Hutchinson and Newton,[27] and Lubimenko and Froloff-Bagreief,[28] believe that long-wave ultra-violet (beyond the lethal limit of approximately 2900 Å) or visible radiation promotes yeast

[18]Feuer, B., and Tanner, F. W., *J. Ind. Eng. Chem.*, **12** (1920): 740.

[19]Schreiber, H., *Strahlentherapie*, **49** (1934): 541.

[20]Tanner, F. W., and Ryder, E., *Botan. Gaz.*, **75** (1923): 309.

[21]Tanner, F. W., and Byerly, J. R., *Arch. Mikrobiol.*, **5** (1934): 349.

[22]Söhngen, N. L., and Coolhaas, C., *Wochschr. Brau.*, **40** (1923): 187.

[23]Abderhalden, E., *Fermentforschung*, **9** (1927): 195.

[24]Norris, R. J., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 57.

[25]Gronchi, V., *loc. cit.*

[26]Guerrini, G., *Boll. soc. ital. biol. sper.*, **5** (1930): 635.

[27]Hutchinson, A. H., and Newton, D., *Can. J. Research*, **2** (1930): 249.

[28]Lubimenko, W., and Froloff-Bagreief, A., *Compt. rend.*, **154** (1912): 226.

growth or fermentation. On the other hand, Wynd and Reynolds,[29] state that ultra-violet between 3000 Å and 3500 Å inhibits the fermentation of yeast. Gronchi[30] claims that "Wood's light" stimulates fermentation, while short ultra-violet affords a temporary stimulation followed by marked depression.

If stimulation by ultra-violet longer than 3000 Å actually occurs, it probably cannot be accounted for by cell injury but must be attributed to a different mechanism. Believing that the wavelength threshold for the stimulation of yeast might be at a longer wavelength than that for the lethal action, and that the use of full ultra-violet might cause a masking of the stimulation by the lethal effect, Loofbourow and Cameron[31] investigated the action of radiations longer than 2900 Å on yeast, but were unable to demonstrate stimulation.

The observation of stimulation by ultra-violet has not been confined to yeasts. Weinstein,[32] Bovie and Hughes,[33] and Alpatov and Nartzukowa[34] have noted that sub-lethal doses of lethal ultra-violet stimulate paramecia in various ways. Hutchinson and Ashton[35] have found stimulation of the cell division of paramecia by visible and near ultra-violet and have observed similar effects on fungi.[36]

The effect of ultra-violet on respiration has received comparatively little attention. Surányi and Vermes,[37] and Tang[38]

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[29]Wynd, F. L., and Reynolds, E. S., *Ann. Missouri Botan. Garden*, 22 (1935): 853.

[30]Gronchi, V., *loc. cit.*

[31]Loofbourow, J. R., and Cameron, J. L., *Bull. Basic Sci. Research*, 5 (1933): 33.

[32]Weinstein, I., *J. Optical Soc. Am.*, 33 (1930): 433.

[33]Bovie, W. T., and Hughes, D. M., *J. Med. Research*, 23 (1918): 223.

[34]Alpatov, W. W., and Nartzukowa, O. K., *Protoplasma*, 18 (1933): 281.

[35]Hutchinson, A. H., and Ashton, M. R., *Can. J. Research*, 1 (1929): 292.

[36]Hutchinson, A. H., and Ashton, M. R., *Can. J. Research*, 3 (1930): 187.

[37]Surányi, G., and Vermes, M., *Magyar Orvosi Arch.*, 30 (1929): 585.

[38]Tang, P. S., *J. Cellular Comp. Physiol.*, 8 (1936): 117.

noted stimulation on microorganisms, and Sonne[39] found a similar effect on the chrysalides of the meal worm. The work of certain other investigators is discussed in a review by Wynd and Reynolds.[40] In this laboratory[41] both stimulation and depression have been demonstrated and explained as already stated.

Some investigators believed that they noted genetic or developmental changes in ultra-violet irradiated cells, indicative of action on their reproductive mechanisms. Thus, there is the work of Mme. Henri[42] on modifications produced by ultra-violet in *B. anthrax* and various fungi, and, more recently, the investigations of Stevens.[43] We have interpreted this work as further evidence that compounds of the cell nucleus play an important part in the action of ultra-violet on cells.[44]

There is evidence for the production of various chemical factors by or in ultra-violet irradiated cells. Burger[45] observed, while working in association with us, that when bacterial smears on agar plates are irradiated with the spectrum from a quartz mercury arc, the lethal lines appear visible in the agar within a short time after irradiation, long before sufficient time has elapsed for appreciable bacterial growth. He attributed this to the production of substances by the irradiated bacteria which act upon the medium. Heilbrunn and Daugherty[46] believe that the effects of ultra-violet on amoeba protoplasm are due to the breaking down of calcium compounds in the cortex. The various photochemical effects

[39]Sonne, C., *Acta Radiologica Scandinavica*, **5** (1926): 419.

[40]Wynd, F. L., and Reynolds, E. S., *loc. cit.*

[41]Fardon, Norris, Loofbourow, Ruddy and Carroll, *loc. cit.*

[42]Henri, Mme. V., *Compt. rend.*, **158** (1914): 1032; **159** (1914): 340.

[43]Stevens, F. L., *Botan. Gaz.*, **86** (1928): 210.

[44]Loofbourow, J. R., and Heyroth, F. F., *Nature*, **133** (1934): 909; *Bull. Basic Sci. Research*, **5** (1933): 13; *Phys. Rev.*, **45** (1934): 126.

[45]Burger, G., *Bull. Basic Sci. Research*, **2** (1928): 46.

[46]Heilbrunn, L. V., and Daugherty, K., *Protoplasma*, **18** (1933): 596.

suggested to explain the lethal action of ultra-violet have been reviewed by Heyroth and Loofbourow.[47] A biochemical basis of the stimulative effects was first suggested, to the best of our knowledge, by Bovie and Hughes,[48] who were of the opinion that it might be due to the collection of small quantities of toxic materials, produced in the cells by the lethal rays. Limited amounts of toxic materials are frequently stimulative, as they point out.

Heyroth and Loofbourow[49] investigated the effects of ultra-violet radiations on various cell purines and pyrimidines and on the nucleic acids. They found two effects: first, an increase in ultra-violet absorption in early periods of irradiation, then a decrease in absorption, indicative of photochemical destruction. They suggested that the early changes in nuclear materials might be associated with the formation of growth-promoting substances and the later changes with the lethal action on cells. In subsequent work, Loofbourow and Heyroth[50] were able to show that destruction of nuclear compounds is of primary importance in the lethal effect of ultra-violet on many types of cells. This conclusion has been confirmed by Oster.[51] The investigations of Loofbourow and Cameron,[52] in which attempts were made to determine whether early products of irradiation of nuclear materials stimulated growth, were, however, inconclusive.

Despite the conflicting results obtained by various workers, the mass of data indicating the stimulation of cells in various ways by ultra-violet could hardly be ignored. We were not

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[47]Heyroth, F. F., and Loofbourow, J. R., *Bull. Basic Sci. Research*, 5 (1933): 13.

[48]Bovie, W. T., and Hughes, D. M., *loc. cit.*

[49]Heyroth, F. F., and Loofbourow, J. R., *J. Am. Chem. Soc.*, 53 (1931): 3441.

[50]Loofbourow, J. R., and Heyroth, F. F., *loc. cit.*

[51]Oster, R. H., *J. Gen. Physiol.*, 18 (1934): 243.

[52]Loofbourow, J. R., and Cameron, J. L., *loc. cit.*

satisfied with the criteria of stimulation or with certain other experimental methods used in our earlier work.[53] It was decided that respiration be used as a means of testing stimulation. Accordingly, the respiration of yeast before and after irradiations with ultra-violet was studied by Fardon,[54] using Barcroft-Warburg manometers. Again, conflicting results were obtained. In Fardon's reported results, it was concluded that the difficulty arose from the fact that in some instances so many of the cells were killed or seriously injured during irradiation that the few survivors were not able to produce as great respiration as the controls, despite the stimulating factors which might be present. Cells irradiated until they no longer respired were, therefore, added to cell suspensions and the respiration of these compared with the controls. When this was done, uniform stimulation was obtained.

In later work, it was found possible to separate from irradiated cells, cell-free fractions which promoted growth and fermentation as well as respiration. These investigations have been summarized in the papers by Fardon, Loofbourow and their co-workers, previously referred to. They are interpreted to indicate (as stated above) that cells injured or killed by ultra-violet or other means produce materials capable of stimulating the metabolic activities of neighboring normal cells.

In these researches, however, it was not established whether cell injury resulted in the release of greater quantities of metabolic stimulating substances through degenerative processes, or whether it stimulated cells to produce the active materials.

In the present investigations we shall endeavor to establish, by easily observable and easily reproducible methods, that the growth-stimulating factors previously investigated are pro-

[53]Loofbourow, J. R., and Cameron, J. L., *loc. cit.*

[54]Fardon, J. C., Carroll, Sr. M. Jordan, O.P., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 35.

duced as a result of cell injury, and that the stimulating effects obtained are not merely those which would be expected from the bios-like substances produced by normal cells or by the disintegration products of cells. Similar investigations are in progress with regard to the respiration, fermentation and glycolytic factors. These will be published subsequently.

EXPERIMENTAL

To accomplish the purposes outlined in the introduction, we endeavored to demonstrate the stimulative action on cultures of cells which could be observed microscopically. *Saccharomyces cerevisiae* from Fleischmann baker's yeast was used because of the ease with which it can be obtained and cultured. Cultures were grown on slants of Sabouraud's medium and transplanted every 24 to 48 hr. Suspensions inoculated from these were used to test the rates of cell proliferation.

For the suspensions subjected to irradiation in the later experiments (hanging block method) 3.5 g. of yeast were taken directly from the interior of a cake of Fleischmann baker's yeast and suspended in 200 cc. of fluid. Ringer's glucose phosphate solution was the dispersing medium except when otherwise noted.

PRELIMINARY EXPERIMENTS

At first, various methods were employed for irradiating a portion of the cells in a particular area on the microscope slide. The rate of budding of the neighboring non-irradiated cells was observed subsequently on a warm stage and compared with the rate of budding of cells in a separate control area, no part of which had been subjected to irradiation.

In some of the experiments, suspensions of yeast in Reader's medium were placed on flat slides, within areas outlined by

Vaseline, and covered with quartz cover glasses. It was difficult to prevent convection currents on these slides. In other experiments, the yeast was suspended in hanging drops of Reader's medium containing gelatin or agar, or in hanging drops of Sabouraud's medium. Each drop was surrounded by an additional ring of agar, covered with a heavy suspension of yeast to be subjected to irradiation. It was difficult in these experiments to arrange matters so that the cells to be irradiated were not shielded by too thick a layer of agar.

Since the materials produced by injured cells appeared from our earlier work[55] to be bios-like, it was thought best to determine if small quantities of bios would stimulate proliferation under the conditions of the experiments. For this purpose, a bios concentrate prepared from malt combings by Kreke,[56] designated by him as *Sample 14*, was used. It had a potency of 10.6 growth units per mg. as determined by his method. This was added to yeast suspensions in various amounts, and the rate of budding on slides on the warm stage compared with the rates of budding of controls. Definite stimulation was obtained in every case.

In the ultra-violet irradiation experiments, the slides were irradiated on the microscope stage, using a Burdick, alternating current, air-cooled quartz mercury arc operated at approximately 65 volts. This was placed immediately above the microscope tube, after the objective and eye piece had been removed. A portion of the area was shielded from irradiation by a drop of mercury or a small copper disc. Since separate series of experiments showed that exposures of about 20 min. at 30 cm. were necessary in order to kill most of the cells, this was adopted as the standard time and distance of irradiation. The methylene blue test was used as a criterion of killing.

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[55]Fardon, Norris, Loofbourow, Ruddy and Carroll, *loc. cit.*

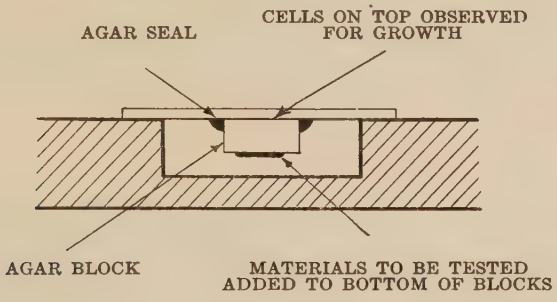
[56]Norris, R. J., and Kreke, C., *this issue*, p. 137.

In all of these preliminary experiments, the results were subject to considerable variation, apparently due to the technical difficulties described above. Stimulation of cell proliferation was obtained, but not consistently.

Hanging Block Method

To overcome such difficulties as the motion of the cells, the shielding of the cells by the medium during irradiation, and the migration of cells from the irradiated into the non-irradiated area, a new method was adopted, employing a hanging block of medium prepared as follows: Agar was added to Reader's

FIG. 1—*Section of Slides used in Hanging Block Experiments*



medium in the proportion of 18 g. per 1000 cc. Reader's medium was chosen because it sustains yeast growth without being too favorable to proliferation. After sterilization, the medium was poured into petri dishes to an approximate thickness of 3 mm. and allowed to cool. Blocks approximately 5 mm. by 5 mm. were cut from the solidified medium and placed on slides. A loopful of yeast suspension, made up in Reader's medium (generally from a 2-day-old slant) was put on top of each block. The slides were placed in petri dishes and put in an incubator at 37° C. for 5 min. to dry the suspension. After removal from the incubator, a cover glass was placed on top of each block, the block lifted from the slide,

and the cover glass turned over. In all but the early hanging block experiments, the block was then sealed to the cover glass with additional agar and Reader's medium placed around the edge, so as to prevent migration of organisms from or onto the top of the block. The material to be tested for its effect on yeast proliferation was then placed on the bottom of the block, and the cover glass inverted and put on a hollow culture slide, ringed with Vaseline to provide an air-tight seal (Fig. 1).

Diffusion of Bios through Agar Blocks

The success of this method depends upon the ability of proliferation-promoting factors to diffuse from the bottom through the agar block to the suspension on top. To test this, Kreke's malt-combings bios, *Sample 14*, was used. Two loopfuls of a 2.6 mg. per cc. solution of this were applied with a calibrated loop of such size that the quantity of material added to the block was approximately 0.0075 mg. The following results (Table I), obtained in observing the rate of budding as compared with that of a control to which two loopfuls of

TABLE I

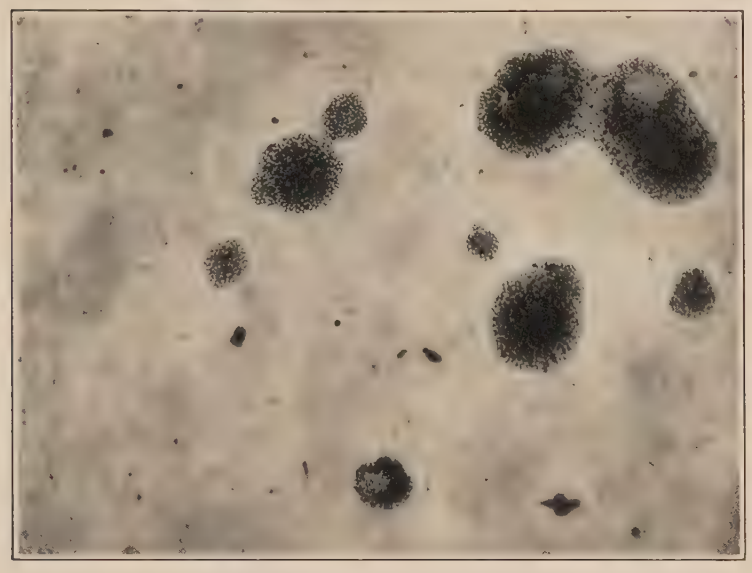
Diffusion of Bios through Agar Blocks

Time	Average Per Cent of Budded Cells in Five Distributed Areas	
	Test Block—Bios Added	Control—Reader's Medium Added
Start 11:40	11	26
2:15	26	26
3:00	50	30
4:50	92	76

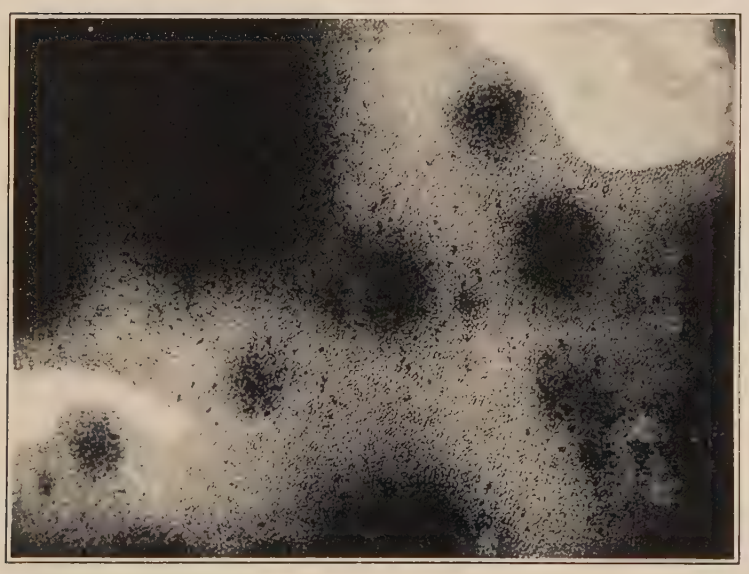
Reader's medium were added to the bottom of the block, show definite stimulation by the bios and demonstrate its ability to diffuse through the block. Both blocks were kept on the warm stage at approximately 33° C. At the end of

PLATE I

Typical microphotographs from experiment 40 showing growth on tops of blocks after 46 hr.



Growth Above Uninjured Cells



Growth Above Ultra-violet Injured Cells

this period of observation, the slides were removed from the warm stage and incubated for 24 hr. at room temperature. There was a considerably greater area covered by growth on the slide to which bios had been added, the ratio of the growth on this slide to that on the control being about the same as that obtained in the irradiation experiments described below.

Proliferation-promoting Effects of Ultra-violet Injured Cells

For determining the production of proliferation-promoting factors by irradiated cells, suspensions of yeast were made in Ringer's glucose-phosphate solution as described above. Samples from these were tested with methylene blue to determine the viability of the cells. If more than a few of the cells were dead, the suspensions were discarded. Approximately 10 cc. from such a suspension was irradiated in a quartz test tube for 40 min., with continual stirring by a motor stirrer, at a distance of 25 cm. from a Burdick air-cooled quartz mercury arc. This time of irradiation was chosen as a result of methylene blue tests on the irradiated suspensions, which showed 40 min. to be sufficient to kill about 90 per cent of the cells. In general, each irradiated preparation was separately tested with methylene blue in order that possible variations in the degree of killing, due to variations in the yeast or the lamp, might be discovered.

A loopful of the irradiated suspension was added to the bottom of each of one or more blocks, and a loopful of the non-irradiated portion of the same suspension to each of one or more control blocks. In most experiments, controls to which nothing was added or a loopful of water added, were also run. In the later experiments, cell-free materials were obtained from the irradiated and non-irradiated cell suspensions by centrifuging them for 15 min. in vaccine tubes and decanting the supernatant fluid. Samples from these were added to blocks as described above.

TABLE II—*Summary of Hanging Block Experiments Showing the Effects of Factors from Injured Cells on Proliferation*

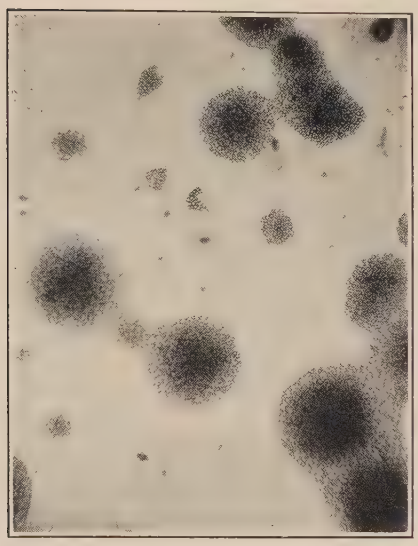
Experiment	Time of Incubation (at room temperature)	Per Cent of Area of Microphotographs Covered with Growth After the Addition of Test Materials					
		Material Added					
		Non-irradiated Cell Suspension	Nothing	Water	Cell-free Fraction from Non-irradiated Cells	Irradiated Cell Suspension	Cell-free Fraction from Irradiated Cells
No. 24	26 hr.	3.7				19.3	
" 25	24 "	5.3				64.6	
" 26	24 "	9.5				34.2	
" 27	48 "	11.3	27.3	6.5		90.0	
" 28	47 "	10.9	34.7			45.9	98.0
" 29	24 "	9.3	8.1	5.3		32.4	29.7
" 30	48 "	*	4.6	14.4		50.0	72.6
" 31	24 "	4.6	6.3	8.6		18.9	34.0
" 32	24 "	7.9	13.4	5.5		22.0	32.9
" 39	27 "	6.5		11.8	14.8	49.2	51.6
" 40	46 "	10.4	18.8	13.7	17.4	90.3	40.5
Averages	24-27 hr. Expts.	6.7	9.3	7.5	14.8	34.3	37.1
		Reader's Medium	Nothing			Bios	
No. 44	26½		18.5			51.6	
" 45	26	6.3	12.4			33.5	
Averages		6.3	15.5			42.6	

NOTE: The significant feature to be observed is the relative growth on the various experimental and control blocks in each individual experiment rather than a similar comparison from experiment to experiment. Uncontrolled variables, such as temperature, operated against uniformity of growth among the different experiments.

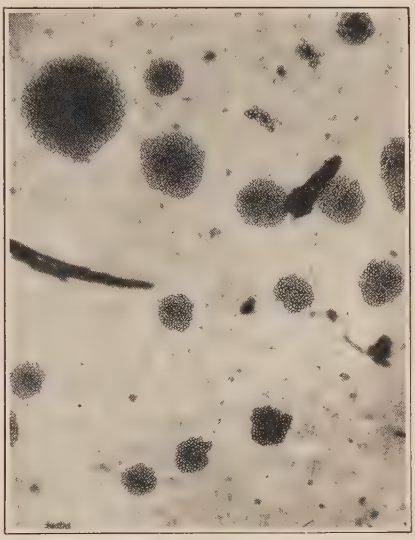
*Could not be photographed due to thin agar.

PLATE II

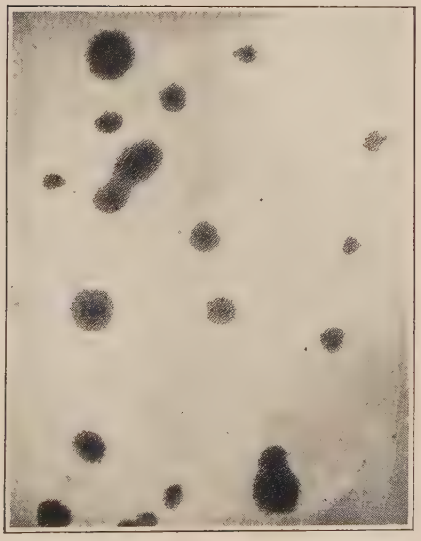
Typical microphotographs from experiment 27 showing growth on tops of blocks after 48 hr.



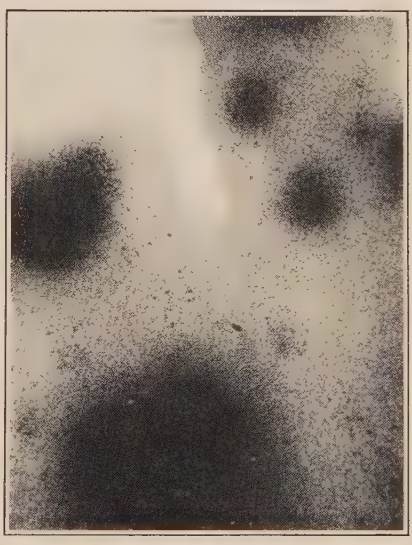
Control—Nothing Added



Control—Water Added



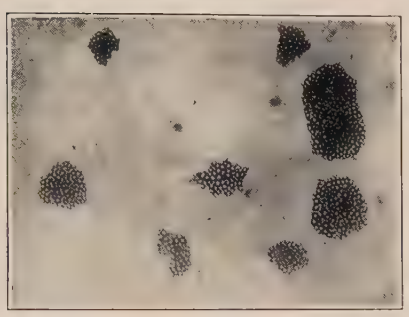
Control—Uninjured Cells Added



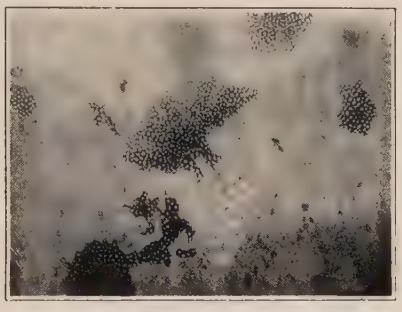
Test—Ultra-violet Injured Cells Added

PLATE III

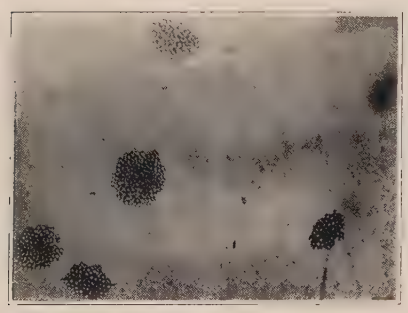
Typical microphotographs from experiment 39 showing growth on tops of blocks after 27 hr.



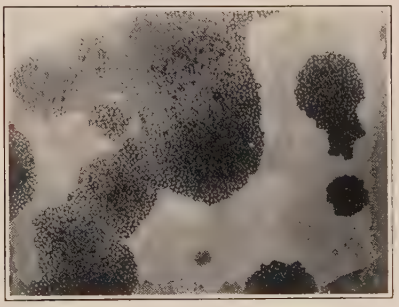
Control—Nothing Added



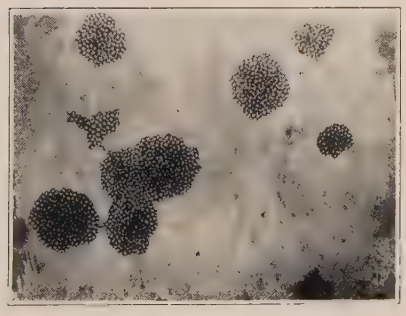
Control—Water Added



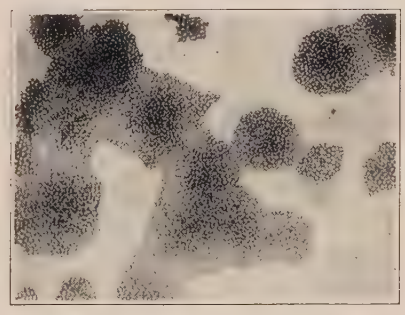
Control—Uninjured Cells Added



Test—Ultra-violet Injured Cells Added



Control—Cell-free Fraction from Uninjured Cells Added



Test—Cell-free Fraction from Ultra-violet Injured Cells Added

The blocks were incubated at room temperature for 24 to 48 hr. and the growth of the cells on top of each block observed microscopically. Microphotographs were taken immediately after observation. Although the procedure is essentially qualitative, estimates of the growth area in each microphotograph were made by dividing the field into 432 squares and counting the number covered with growth. Table II summarizes the results on the basis of these area measurements. The accompanying microphotographs have been selected from a large number as typical.

From the table, it is apparent that the addition of ultraviolet injured cells to the bottoms of the blocks produced marked stimulation of cell proliferation in every case as compared with the various controls. In most instances, the cell-free fractions from the irradiated cells produced greater stimulation than the irradiated suspensions, indicating that the proliferation-promoting factor is something which is secreted by the cells into the surrounding medium. This agrees with our earlier results.[57]

Comparison of the growth areas on blocks to which irradiated cells or irradiated cell-free fractions had been added with those on blocks to which non-irradiated cell suspensions had been added, shows that the effect is not merely one resulting from the production of bios-like substances by normal cells. Indeed, the blocks to which non-irradiated cell suspensions were added generally showed less growth than controls to which nothing had been added, a result which is probably explained by the lesser amount of nutrient material available per cell and the greater accumulation of waste products in the former case.

A further indication that the proliferation-promoting factors arise from injured rather than normal cells is found in com-

[57]Fardon, Norris, Loofbourow, Ruddy and Carroll, *loc. cit.*

paring the growth produced by cell-free fractions from irradiated and non-irradiated suspensions with the growth of the controls. The cells were subjected to some mechanical injury in centrifuging, and the cell-free fractions from the suspensions, centrifuged but not irradiated, showed some proliferation promotion as compared with the controls but not as much as that shown by the cell-free fractions from irradiated suspensions.

TABLE III

Comparison of the Effects of Centrifuged and Filtered Cell-free Fractions from Non-irradiated Suspensions

Samples	Time	Per Cent of Area Covered with Growth	
		Non-irradiated Cells	Cell-free Fractions from Non-irradiated Cells
Centrifuged:			
Exp. 39	27 hr.	6.5	14.8
Exp. 40	46 hr.	11.1	16.5
Filtered:			
Exp. 37	26 hr.	17.2	64.5
Exp. 78	26 hr.	3.7	52.0

In a few experiments, cell-free materials were prepared by vacuum filtration through Berkefeld filters. One might expect somewhat greater mechanical injury to the cells in this case, and it is interesting to note, from the typical results shown in Table III, that greater proliferation promotion was apparently obtained from the cell-free fraction from non-irradiated suspensions in these instances than from such fractions obtained by centrifuging.

Nature of the Proliferation-promoting Factor

Whether the cell-proliferation-promoting factor (or factors) produced by the injured cells is of the nature of a cell hormone

or vitamin or of the nature of ordinary nutrient material can best be answered by a consideration of the quantities of materials added to the blocks in the various experiments, together with the quantity of nutrient material contained in each block. For the purpose of comparing these quantities, the yeast suspensions for irradiation were always made up in known concentrations by weight, as described above, and the dry weights of materials in the cell-free fractions were determined by evaporating samples of these materials to dryness and weighing them on a sensitive analytical balance. The comparative weights are shown in Table IV.

These data show that the dry weights of the materials added to each block (after subtracting the dry weight of the Ringer's solution in which the cells were suspended) ranged from 0.0011 to 0.025 of the weight of dextrose in the agar block. In the case of the irradiated cell-free fractions, the weights of the material added were of the order of one-thousandth to three-thousandths of the weight of dextrose in the block. That such minute quantities of materials from cells injured by ultra-violet have proliferation-promoting power indicates that their action is hormone-like rather than that they are simply supplying additional nutrient material for the cell cultures. This is further borne out by the fact that the cell-free fractions from irradiated cells stimulated proliferation to about the same extent as Kreke's bios, *Sample 14*, when employed in quantities only about one-half as great as the quantities of bios used.

It should be noted that the weight of the materials (other than Ringer's solution) added to each block from the cell-free fractions of the irradiated cells was only about one-seventh as great as the weight of the materials added from the irradiated cell suspensions, despite which the cell-free fractions produced greater stimulation than the cell suspensions. This affords further confirmation that the cell-proliferation-promoting ma-

TABLE IV

Weights of Materials Added to Blocks

Average Weights of Materials	A Total Weight	B Agar	C Dextrose	D Other Salts	E Ringer's Solution	F Materials other than Ringer's Solution	G Ratio *F/C*
Block	0.0033 g.	0.0018 g.	0.001 g.	0.0005 g.			
1 loopful of yeast suspension (irradiated or non-irradiated)	0.0000382 g.				0.0000132 g.	0.000025 g.	0.025
1 loopful of cell-free fraction from irradiated cells	0.000017 g.				0.0000132 g.	0.0000038 g.	0.0038
1 loopful of cell-free fraction from non-irradiated cells	0.0000143 g.				0.0000132 g.	0.0000011 g.	0.0011
2 loopfuls of Kreke's bios *Sample 14*	0.0000075					0.0000075	0.0075

terials produced by injured cells are eliminated from the cells into their suspending medium.

Relation of the Production of the Active Materials to Injury

If the proliferation-promoting factors are produced by cells during the process of injury, one might expect the efficacy of their production to depend upon the metabolic state of the cells. Thus, cells suspended in various kinds of dispersing media might be expected to produce different amounts of proliferation-promoting materials under comparable conditions of irradiation.

In preliminary experiments, using cultures grown in rocker tubes as well as the hanging block technique, we have found that cells irradiated in distilled water have little proliferation-promoting activity. Most of the cells in these suspensions were dead before irradiation (as determined by the methylene blue test). The conditions were favorable to the production of dead-cell disintegration products, yet little proliferation promotion resulted.

Cells suspended in dilute alcohol during irradiation likewise resulted in practically impotent preparations. Yet dilute alcohol is commonly employed in the extraction of "bios" from yeast. If the proliferation-promoting factors were simply the bios-like substances of normal cells set free by irradiation, one would expect irradiation under these conditions to lead to potent materials.

On the other hand, cells irradiated in isotonic sodium chloride solution, Ringer's glucose-phosphate solution, or Ringer's solution without glucose resulted in materials of uniformly high activity, provided the conditions of irradiation were such as to kill approximately the same number of cells (90 per cent) in the same length of time (40 min.). Under these circumstances, the cells remained mostly viable

up to the time of irradiation, and the only injurious agent to which they were subjected was the ultra-violet radiation, the intensity of which was sufficiently low so that, in general, prolonged injury took place before death. The fact that the most potent preparations were obtained under such conditions indicates that the active factors are elaborated by living cells as a physiologic response to injury.

This conclusion is further borne out by experiments in which the time and intensity of irradiation were varied in such a way as to keep the percentage of cells killed approximately the same. When high intensities were used, so that 90 per cent killing was obtained in a short time, the potencies of the resulting materials were less than when low intensities, requiring long exposures for the same degree of killing, were employed.

These preliminary experiments are being extended, using rocker-tube methods to establish quantitative data. The results will be published subsequently.

Comparison of the Effects of Lethal and Non-lethal Ultra-violet

The results of those workers, referred to in the review of the literature, who found long-wave ultra-violet, beyond the limit of the lethal spectrum, to stimulate cell-proliferation, would seem to indicate that proliferation-promoting factors can be produced by irradiation with non-injurious rays. Loofbourow and Cameron[58] failed to find such results, however, and we may briefly refer here to some further experiments conducted in 1934 by Loofbourow and Helmer, but never published. In these experiments, yeast suspensions in a thin molasses medium were irradiated in rotating quartz cylinders in an irradiating equipment devised by Goode[59] by means of

[58]Loofbourow, J. R., and Cameron, J. L., *loc. cit.*

[59]Goode, P., *Bull. Basic Sci. Research*, 5 (1933): 39.

which the yeast could be exposed to filtered ultra-violet of fairly sharp wavelength limit. The time of irradiation was uniformly 1 hr., and the filters used had "absorption limits" of 2890 Å and 2750 Å (as defined in Goode's paper). Control suspensions were kept at the same temperature as those in the quartz cylinder during the period of irradiation. Samples from the molasses suspensions were plated out immediately after inoculation and again 3 hr. later. Comparison of the counts on plates made before and after the standing period was used as a criterion of proliferation stimulation. Under these conditions, the controls showed no proliferation, nor did the samples irradiated through the 2890 Å filter, while those irradiated through the 2750 Å filter showed uniformly slight, but noticeable, proliferation, as illustrated in Table V.

TABLE V

Ultra-violet Irradiation of Yeast Suspensions

	Average Plate Count
Plated immediately after inoculation	138
Plated 3 hr. later:	
Non-irradiated samples	138
Irradiated through 2890 Å filter	134
Irradiated through 2750 Å filter (which killed some cells)	147

Irradiation through the 2890 Å filter for prolonged periods under the conditions of these experiments failed to kill yeast, while irradiation under the 2750 Å filter killed some cells and hence must have injured many. These results indicate that the long-wave ultra-violet, of such spectral region as not to injure the cells, does not produce proliferation-promoting materials of the kind noted in our work. The results cannot be considered more than indicative, however, and the whole

matter needs to be reinvestigated with great care. The newer techniques, in which cell-free materials are separated from the irradiated cells and added to other cell suspensions, affords a better means of testing the matter.

If there is any stimulation of growth by radiation of longer wavelength, it may be due to an entirely different mechanism than the one which we believe to be acting in the case of cell injury.

Ultra-violet Absorption Spectra

Ultra-violet absorption spectra of cell-free fractions from irradiated and non-irradiated cell suspensions were determined by means of a Spekker photometer and a Hilger quartz spectrograph. Fig. 2 shows examples of such spectra. The absorption of the cell-free fractions from irradiated cells was considerably greater than that of the fractions from non-irradiated cells. A distinct band at 2600 Å is apparent in both spectra, typical of nucleic acid and certain of its constituent purines and pyrimidines.[60]

The band at 2750 Å in the material from non-irradiated cells (which is evident to a lesser extent in the material from irradiated cells) is suggestive of tyrosine or tryptophane.

Of all the purines and pyrimidines we have studied, the spectrum of yeast nucleic acid[61] resembles most closely the spectrum of the irradiated material. This is interesting in view of the suggestion of Mathews[62] that abnormally great formation of nucleic acid may be the mechanism which leads to rapid cell division in cancer tissue. The quantitative values of absorption are such that one cannot attribute it to nucleic

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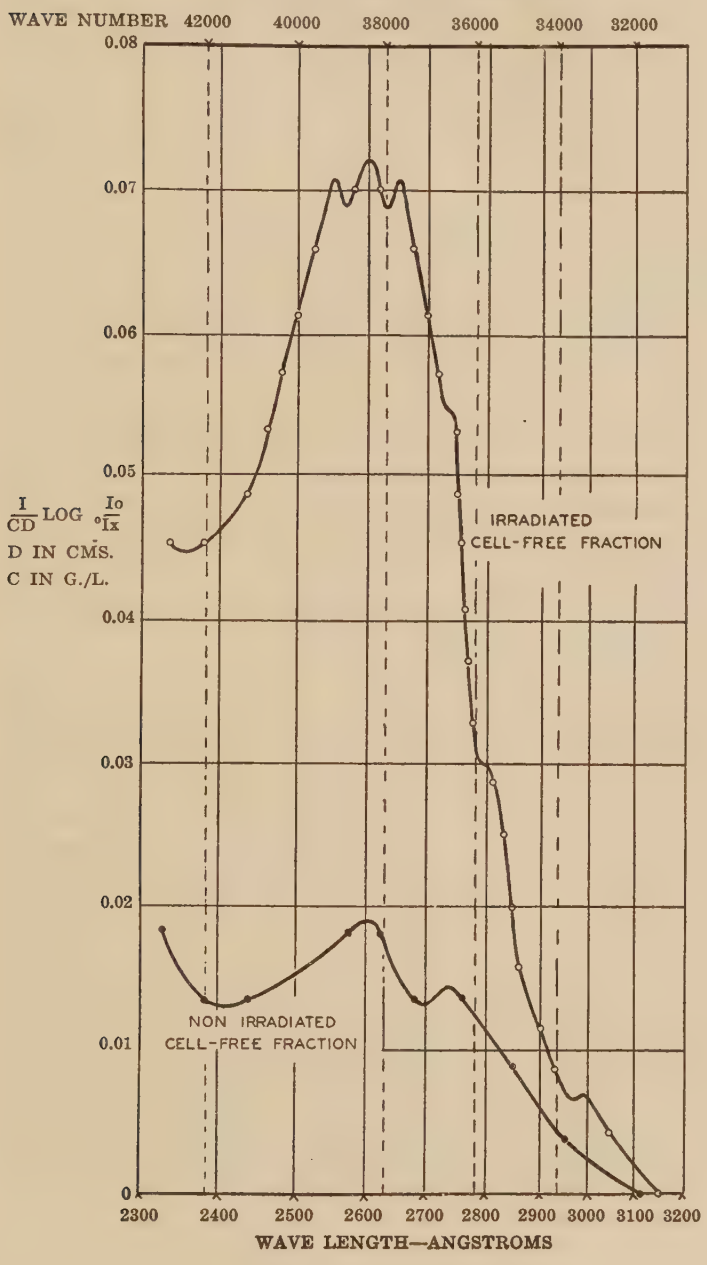
[60]Heyroth, F. F., and Loofbourow, J. R., *J. Am. Chem. Soc.*, 56 (1934): 1728.

[61]Heyroth, F. F., and Loofbourow, J. R., *Bull. Basic Sci. Research*, 3 (1931): 237.

[62]Mathews, A. P., Principles of Biochemistry, Wm. Wood & Co., (1936).

FIG. 2 — *Ultra-violet Absorption Spectra of Cell-free Fractions from Yeast Suspensions*



acid alone. The detailed discussion of the spectra is beyond the scope of the present paper, however. It will appear elsewhere.

CONCLUSIONS

The results of these experiments may be summarized as follows:

1. Cells injured by ultra-violet produce materials which stimulate cell proliferation. This confirms our previously reported findings.

2. The proliferation-promoting effects of ultra-violet irradiated yeast are not due merely to substances found in the normal cell suspensions.

3. The factors from irradiated cell suspensions are effective in as small quantities as highly purified preparations of "bios."

4. The proliferation-promoting factors from irradiated cells are effective in such small quantities as to suggest that their action is hormone-like.

5. The active materials are apparently produced by living cells as a result of cell injury.

The significance of these findings with regard to wound healing is apparent, as suggested previously.[63] It is recognized that cells which live together in close proximity influence each other through the products of their metabolism. It is reasonable to suppose that there should be mechanisms which would permit cells to react on one another in such a way as to enable the cell community to respond to sudden changes in conditions (as, for example, the destruction of a part of the community) so as to preserve community life. One would expect such mechanisms to be biochemical.

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[63] Fardon, Norris, Loofbourow, and Ruddy, *loc. cit.*; Sperti, G. S., Loofbourow, J. R., and Dwyer, Sr. Cecilia Marie, S.C., *Nature*, **140** (1937): 643; Sperti, G. S., Loofbourow, J. R., and Lane, Sr. M. Michaella, S.C., *Science*, in publication.

The importance of tissue products in the proliferation and growth of tissue cells has been emphasized in Carrel's pioneering studies of tissue cultures.[64] He gave the name "trephones" to growth-promoting products from cells and tissues which he believed to act as essential foodstuffs rather than as hormones. Wiesner[65] as long ago as 1892 suggested the production of wound hormones by injured plant tissues. Wildiers[66] stated in 1901 that yeast cells produce a substance, "bios," necessary for the proliferation of yeast. Since this early work, many investigators have turned to the study of growth inhibiting and growth stimulating substances from cells and tissues.

All of these investigations seem to us to take on new meaning in the light of our studies. For now we are able, for the first time, to show not simply that there are proliferation-promoting and inhibiting substances present in tissues and in cell cultures, but that proliferation-promoting substances are elaborated by injured, living cells as a definite response to injury. By the new techniques described in this and the previous papers, it is possible, for the first time, to produce such substances at will from many kinds of cells and tissues.

There is considerable evidence in the literature relating injured tissues to the processes of repair. This work has been reviewed adequately by Arey,[67] and we need not discuss it here. There is also evidence that lethal ultra-violet promotes wound healing under appropriate circumstances[68] and that foreign bodies, dirt, and other agents which injure cells lead to faster healing. The fact that we are now able to produce cell-free proliferation-promoting materials at will by injuring

[64]Carrel, A., *J. Am. Med. Assoc.*, 82 (1924): 255; *Physiol. Rev.* 4 (1924): 1.

[65]Wiesner, J., Elementarstructur, Vienna, (1892).

[66]Wildiers, E., *La Cellule*, 18 (1901): 313.

[67]Arey, L. B., *Physiol. Rev.*, 16 (1936): 327.

[68]Arey, *loc. cit.*, Laurens, H., The Physiological Effects of Radiant Energy, Chem. Cat. Co., (1933).

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cells, combines with all of this indirect evidence to point to the elaboration of metabolic stimulating materials by injured cells as the fundamental mechanism of the response of cell communities to injury.

In investigations reported elsewhere,[69] we have demonstrated the stimulation of the growth of tissue cultures of chicken heart by cell-free materials from injured cells. We have recently extended this work to cultures of other kinds of tissues. Details of these investigations will appear in due course. Because of these results, we feel justified in being more positive in our conclusions than we could be were it necessary for us to rely upon the results with yeast alone.

It should be noted that we are dealing in this discussion with injured cells as distinguished from injured multicellular organisms. It is injury to the individual cells in a culture or tissue which appears to lead to the production of the factors we have isolated, rather than gross injury to the organism.

Cell injury may or may not result from injury to the organism. Thus, a clean cut injures comparatively few cells. A burn destroys the majority of cells affected by it. Our work indicates that the active factors are metabolic products of live, injured cells rather than disintegration products of dead cells. Consequently, it may very well be that the stimulation to repair in such tissue injuries as clean cuts and burns, in which many cells may be destroyed but few injured, comes largely from leucocytes, as suggested by Carrel,[70] rather than from injured cells of the tissues affected.

In the case of benign and malignant tumors, it seems reasonable to postulate that the fundamental mechanism involved is the collection in abnormal concentration of metabolic

[69]Sperti, Loofbourow and Lane, *loc. cit.*

[70]Carrel, A., *loc. cit.*

stimulating factors produced by cells as a result of irritation or injury of long standing.

Study of the etiology of tumors points so often to prolonged injury or irritation as the cause, that one has but to refer to any standard pathology for a discussion of this subject. The injurious agencies (such as x-rays) which produce malignant growths are typical causes of injury to large volumes of cells. Under such circumstances, one would expect the neighboring normal cells to be subjected to a considerably higher concentration of proliferation-promoting factors than in instances of ordinary wounds, such as cuts and burns. The surface area of a spherical cell aggregate increases as the square of the radius whereas the volume increases as the cube. When large volumes of cells are injured, the surface of the injured area is less in proportion to the volume than when small volumes are injured. This, and the fact that in ordinary wounds most of the affected cells are destroyed and comparatively few merely injured, seems to explain why ordinary wounds do not usually become malignant whereas large volumes of cells injured rather than destroyed over a prolonged period of time frequently do.

These matters, we feel, are more fittingly discussed in a paper describing animal experiments than in one dealing with yeast. As the results of such experiments will soon be published, we prefer to defer further consideration of the matter until the appearance of that work.

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SPECIFICITY OF BIOS PREPARATIONS FOR BACTERIA

By JOHN R. LOOFBOUROW, ROBERT J. NORRIS, and SISTER M. NORBERT MORGAN, R.S.M.

In the study of stimulating factors obtained from injured cells,[1] we were interested in determining if these factors are related to such stimulating materials as bios. Superficial similarities between such factors and preparations of bios from malt combings have been observed in this laboratory.[2] Our recent results indicate that there are several stimulating factors obtainable from injured cells. Hence it seemed advisable to investigate by similar criteria the question of the multiple nature of bios.

The method used in the following investigations was a biological one, in which the proliferation-promoting activity of four preparations of bios was tested on six strains of bacteria. The results are summarized in the accompanying table.

The yeast bios preparation was made by the method of Narayanan,[3] and corresponded to his fraction "d." The malt-combing preparations were made by Kreke[4] following the methods of Lucas.[5] Kreke's *Sample 8* corresponded to Lucas' filtrate from the lead acetate-ammonia precipitation, and Kreke's *Sample 14* to Lucas' acetone precipitate.

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[1]Fardon, J. C., Norris, R. J., Loofbourow, J. R., and Ruddy, Sr. M. Veronita, O.P., *Nature*, **139** (1937): 589; Fardon, J. C., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 41.

[2]Norris, R. J., and Ruddy, Sr. M. Veronita, O.P., *this publication*, **1** (1937): 53; Loofbourow, J. R., Schmieder, L., Stimson, Sr. Miriam Michael, O.P., and Dwyer, Sr. Cecelia Marie, S.C., *ibid*, **1** (1937): 79.

[3]Narayanan, B. T., *Biochem. J.*, **24** (1930): 6.

[4]Norris, R. J. and Kreke, C., *this issue*, p. 137.

[5]Lucas, G. H. W., *J. Phys. Chem.*, **28** (1924): 1180.

TABLE I

Summary of Experiments on the Effects of Bios on Bacteria

Organism	Bios Preparation (0.1 to 2.0 cc. in 5 cc. total volume).			
	Authors' Preparation from Yeast. 5.2 Growth Units per mg. Used at 43.4 mg. per cc.	Kreke's *Sample 8* from Malt Combings. 0.38 Growth Units per mg. Used at 50 mg. per cc.	Kreke's *Sample 14-1** from Malt Combings. 10.6 Growth Units per mg. Used at 33 mg. per cc.	Kreke's *Sample 14-2** from Malt Combings. Used at 33 mg. per cc.
Staphylococcus aureus	Marked stimulation	Slight stimulation	Marked stimulation	Marked stimulation
Haemolytic streptococcus	Marked stimulation (Hartley broth)	Slight stimulation (Hartley broth)	No stimulation (Nutrient broth)	Marked stimulation (Hartley broth)
Aerobactor aerogenes	Inhibition	Inhibition	Inhibition	Inhibition
Alcaligenes faecalis	No stimulation	No stimulation	No stimulation	No stimulation
Bacillus pyocyaneus			No stimulation	
Diplococcus pneumoniae			No stimulation	

**Samples 14-1* and *14-2* are the same fractions from two identical fractionation processes.

The bacteria were grown in broth and the bios preparations added in the amounts shown. Counts were made by a photoelectric method, by plating, by centrifuging in vaccine tubes, and by counts of stained smears. Each preparation was tested several times on each organism with the exception of *Bacillus pyocyaneus* and *Diplococcus pneumoniae* on which malt combings *Sample 14-1* alone was tested. The results were consistent within the limits of experimental error necessarily accompanying the methods employed.

The table shows that the effects of the preparations on the different strains of bacteria ranged all the way from marked stimulation to inhibition. In general, there was a parallelism in the effects of the different preparations, except that Kreke's *Sample 14-1* gave no stimulation on *streptococcus* whereas the other preparations all did. This may have been due to the use of nutrient broth, instead of Hartley broth, as a culture medium for *streptococcus* in this one instance. Because of the parallelism in the effects of the different preparations, a multiplicity of active factors in them was not indicated.

These experiments do not, of course, preclude the existence of several proliferation-promoting factors for bacteria in bios preparations. They show only that the particular preparations tried have almost parallel effects on several microorganisms. Further work is in progress in which a wider variety of bios preparations and preparations of proliferation-promoting factors from injured bacteria and other cells are being tried on a more extended selection of microorganisms, using quantitative methods of evaluating the potencies of the preparations. Full details of this work will be published later.

REFLECTION SPECTRA OF EYE PIGMENT IN DROSOPHILA MELANOGASTER AND ITS MUTANTS

JOHN C. FARDON AND SISTER MARY JORDAN CARROLL, O.P.

INTEREST in the relation of genes to eye-color development in *Drosophila melanogaster* led to an attempt to determine the difference between the eye pigments of the wild-type fly and mutant eye colors. Although a number of pairs of genes are held to cooperate in the formation of eye color, one pair seems to control the eye color of mutants. This, at first, led observers to think of the pigments as being distinctly different. The possibility was suggested as early as 1913 by Morgan and Bridges.[1] Johannsen,[2] from microscopic examination of sections fixed in alcohol, concluded that there are two pigments responsible for eye color, i.e., wine-red and ochre-yellow. However, in another part of this same paper he indicated that this might not be the case as can be concluded from his statement which follows: "A spectrum analysis of crushed heads of several of the mutants shows no difference in the elements present, though there is some difference in the intensity of some of the lines. Experiments along these lines and in the chemical analysis of the pigments have not been carried far enough to draw very definite conclusions."

"Diluters" and "intensifiers" have long been the terms used to designate the factors which produce the effect of giving lighter or darker shades to eye color and body color in *Drosophila*. This would indicate that these researchers considered only one pigment to be responsible for all eye colors. Ford and Huxley[3] after extensive work on the genes and rates

[1]Morgan, T. H., and Bridges, C. B., *J. Exptl. Zool.*, 15 (1913): 429.

[2]Johannsen, O. A., *J. Morphol.*, 39 (1924): 337.

[3]Ford, E. B., and Huxley, J. S., *Brit. J. Exptl. Biol.*, 5 (1927): 112.

of development in *Gammarus chevreuxi* suggested that "the effects of multiple allelomorph series (e.g., for the eye color of *Drosophila*) may represent a cross-section through a series of developmental curves of one and the same substance, the curves differing as regards rates of formation of the substance, times of onset of deposition, and final level of the equilibrium position obtained."

Visual microscopic observations may readily lead one to the conclusion that mutant eye colors represent merely variations in concentration of one pigment. Such visual observations, however, are highly unsatisfactory and unreliable, and for this reason spectroscopic means were employed to settle definitely whether different pigments or different concentrations of the same pigment are responsible for the different eye colors of *Drosophila melanogaster* and its mutants.

The absorption spectra of the red-eyed pigment have been determined by Laki[4] and the maximum found to be at 8000 Å. He extracted the pigment with water by grinding with sand. His conclusions were that the different eye colors seemed to be one and the same pigment and that the red or yellow color was due to either a change in pH or the oxidation of the pigment. Since Laki worked with only one pigment (pigment of the wild-type) this did not give sufficient data to warrant definite conclusions. Van Atta and Van Atta[5] compared, by the use of a dispersion spectrograph, the reflection spectra of the eyes of red and mutant dilute red, which they state, "is a dominant eye color mutation obtained by radium irradiation." They concluded that "the effect of Dilute on the Red eye is a reduction of reflected light in two portions of the spectrum, the red and the blue." In our work spectra of not only the red but of widely varying and distinctly different mutant eye colors were studied. To eliminate any changes

[4]Laki, K., *Magyar Biol. Kutatóintézet Munkái*, 8 (1935-36): 356.

[5]Van Atta, E. W., and Van Atta, L. C., *Am. Naturalist*, 65 (1931): 382.

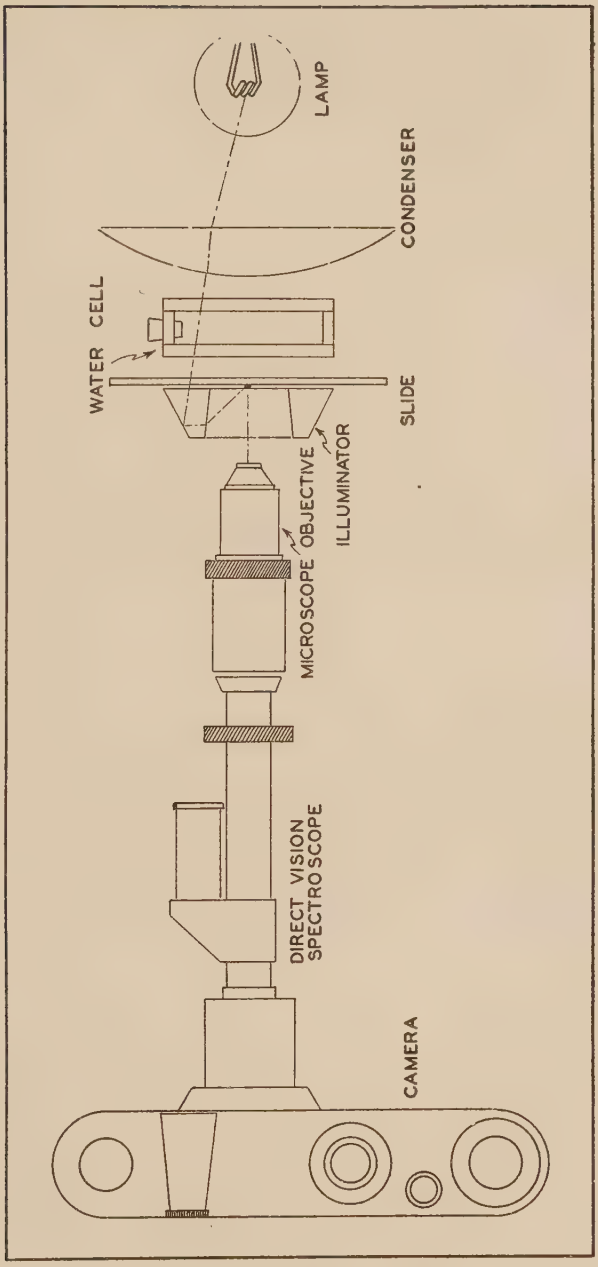

FIG. 1—*The Photospectrograph*

which might take place during extraction, a modified procedure of Van Atta was followed, in which the reflection spectra of the eye were taken while it was still intact rather than spectra of the extracted pigment.

These reflection spectra were obtained by means of the apparatus shown in Fig. 1. The fly was glued to a microscope slide so that a full view of the compound eye was obtained. The slide was placed against a circular prismatic illuminator,[6] and the light so adjusted as to give a uniform field of illumination over the entire convex surface of the eye. A water cell was placed between the illuminator and the condensing lens to absorb the heat radiations. A 16 mm. microscope objective focused the image of the eye upon the slit of a small direct-vision spectroscope, which in turn was screwed on a camera in place of the lens. A series of exposures, taken to determine which would give best results for spectroscopic determinations, revealed that an exposure of 25 min. was most satisfactory.

Reflection spectra of each eye were taken together with reflection spectra from a standard white surface. In these preliminary experiments the standard surface was a sheet of white paper. The reflection spectra from the white surface served as a means to correct any major variations in film sensitivity for the various wavelengths and any differences in emission spectra of the source, which was an incandescent tungsten filament. The film used was Eastman Super X Panchromatic (Type C Emulsion). The sensitivity curve for this film in the visible region (as determined by the Eastman Kodak Co.) is given in Fig. 2. It may be seen from the curve that the film gives good sensitivity to all radiations including the red. All films were developed under similar conditions to insure uniformity.

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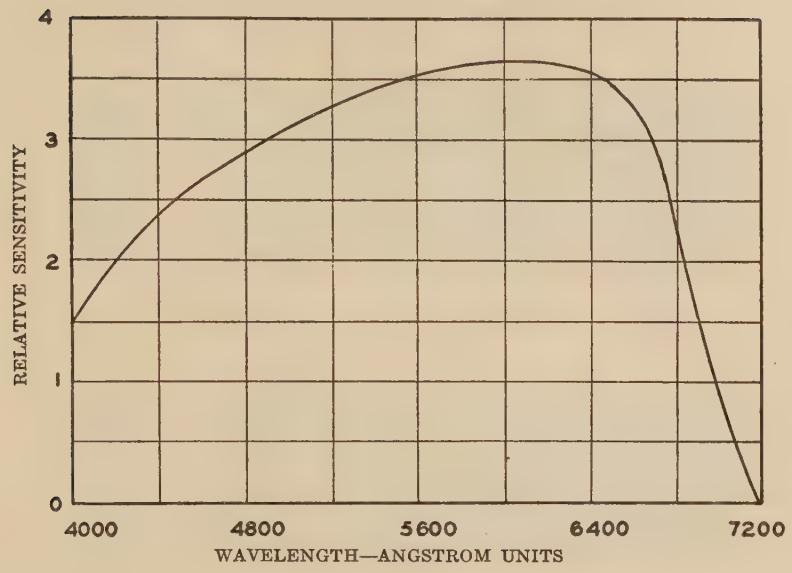
[6]Beck, W. A., *Transactions of the American Microscopical Society*, 42-43 (1923-24): 108.

FIG. 2—*Sensitivity Curve of Type C Emulsion*



To measure the densities of the various regions of the photographed spectra, a simple densitometer was constructed as shown in Fig. 3. The instrument consisted of a film track, a slit adjusted to a width of 0.25 mm., an eye-piece for aligning the wavelength scale with the slit, a source of constant illumination, a condensing lens, and a Weston Photronic cell. The Photronic cell was connected to a sensitive D'Arsonval galvanometer. The film was passed over the slit and galvanometer readings taken at intervals of 100 to 200 Å through the entire visible region of the spectrum.

Prints of the white paper (A), white eye (B), red eye (C), and sepia eye (D), are shown on Plate I.

Fig. 4 and 5 show the curves for the eye colors of males and females respectively. All the eye colors investigated have their maximum reflection in the neighborhood of 6600 Å. In general, there is not much difference in the curves of males and females of the same eye color. As is to be expected the white-

FIG. 3—*The Densitometer*

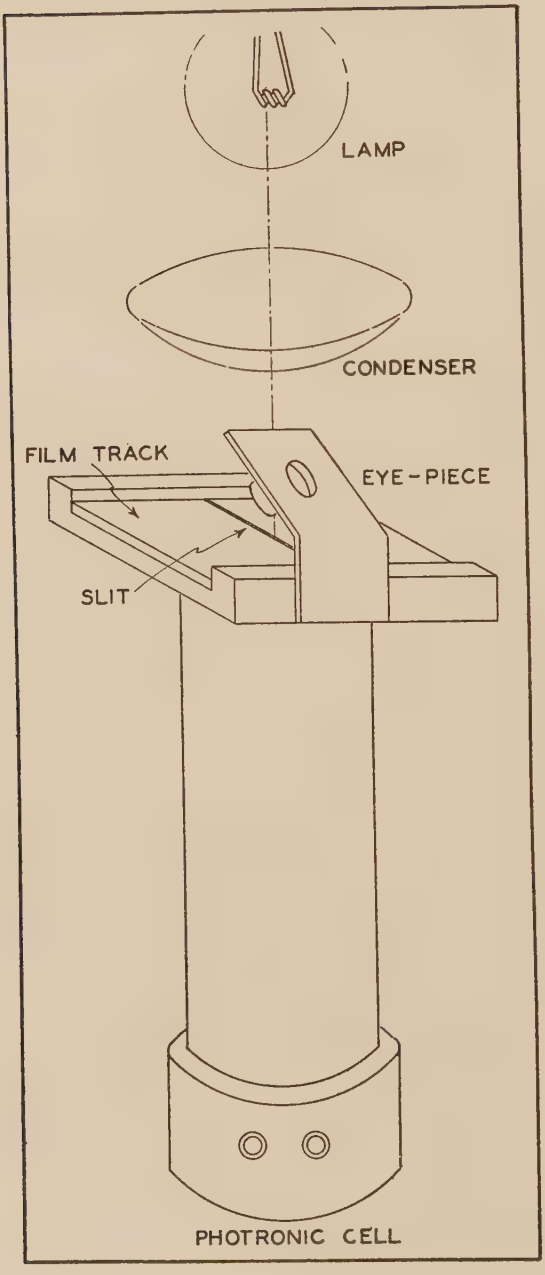
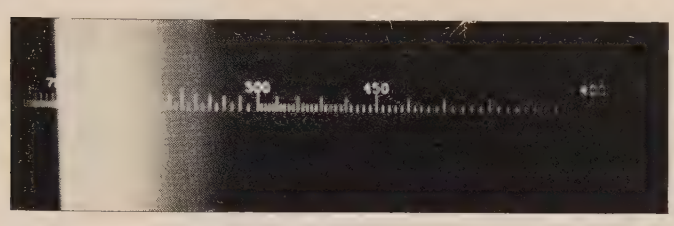
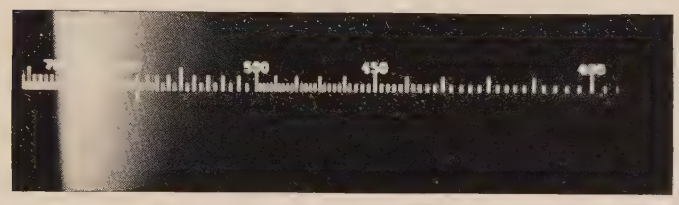


PLATE I

Reflection Spectra of Eyes of Drosophila Melanogaster and its Mutants



Reflection Spectrum of Standard (A)



Reflection Spectrum of White Eye (B)

Reflection Spectrum of Red Eye (C)

Reflection Spectrum of Sepia Eye (D)

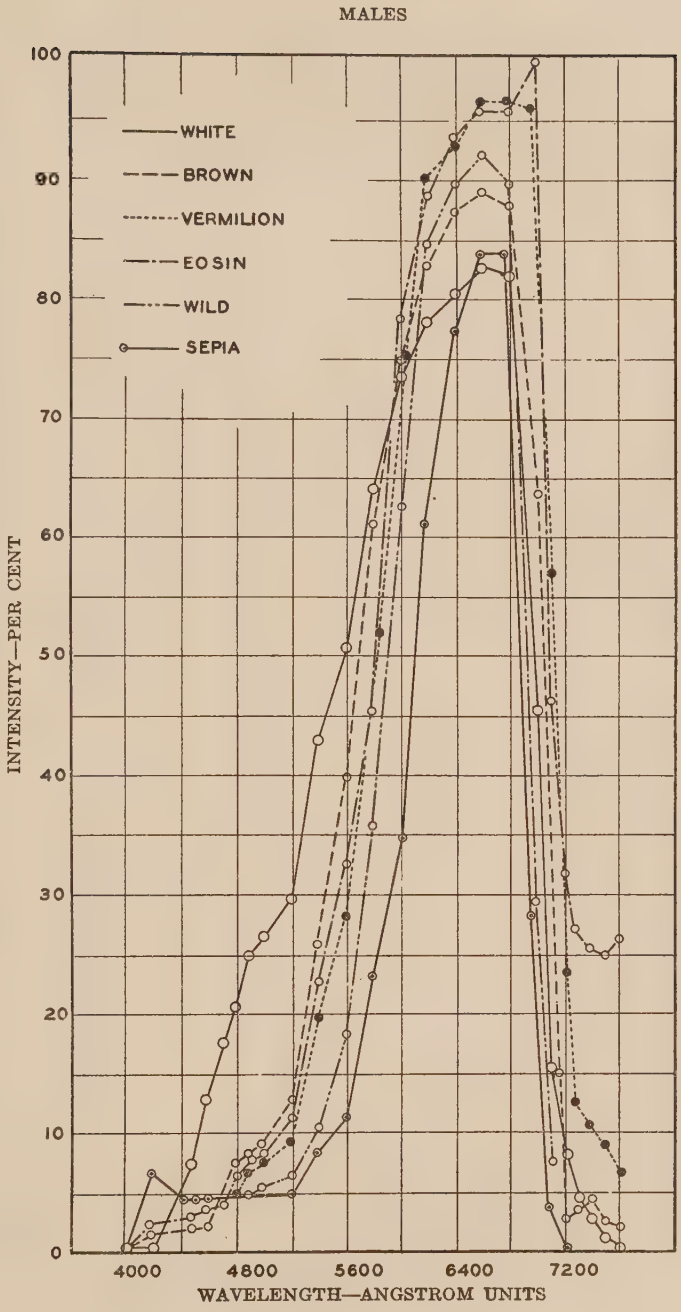


FIG. 4—*Curves of the Reflection Spectra of the Eyes of Drosophila Melanogaster and Its Mutants*

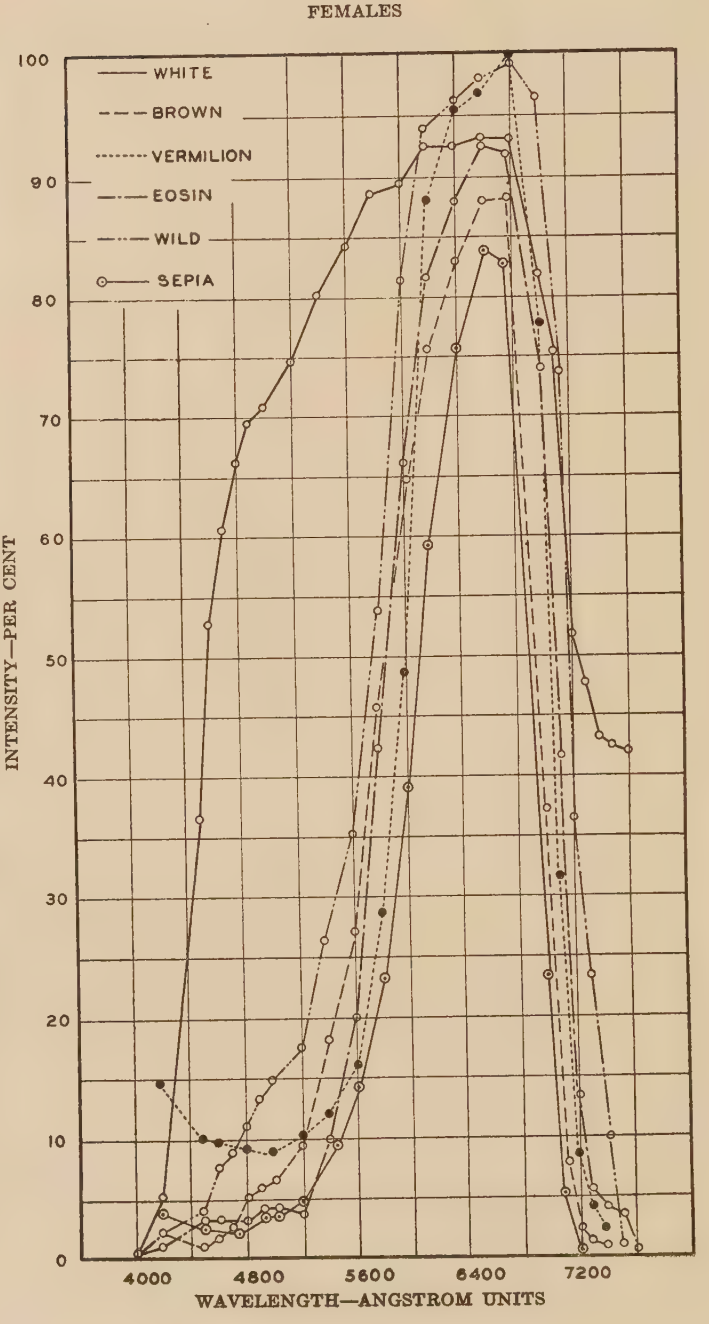


FIG. 5—*Curves of the Reflection Spectra of the Eyes of Drosophila Melanogaster and Its Mutants*

eyed flies show intense reflection throughout the entire visible portion of the spectrum, while reflection spectra of the sepia-eyed flies show a minimum reflection covering only a band of about 300 Å. Furthermore, it will be noted that all the other eye colors are envelopes of the sepia.

CONCLUSIONS

From an analysis of the reflection spectra of the eyes of *Drosophila melanogaster* and some of its mutants it may be concluded that all the eye colors investigated by the authors are dilutions of sepia color. Although a more refined spectroscopic analysis may show differences not revealed by the present technic, it does seem reasonable that only one pigment is involved and that its concentration is determined by the mutant and normal genes which enter into the germ plasm under a set of given conditions. The bearing of these results on the present genetic theories will be discussed in a future paper.

VITAMIN D PRECURSORS REMOVED FROM HUMAN SKIN BY WASHING

By AGNES C. HELMER and REV. CORNELIUS H. JANSEN

In the early experiments[1] on the identification of vitamin D and its precursors, it was found that cholesterol, and later ergosterol,[2] irradiated with ultraviolet, acquire antirachitic properties. Cholesterol is found in great abundance in the skin of humans as well as animals. Eckstein and Wile,[3] who have investigated carefully the cholesterol content of human skin, found that an average of 19 per cent of the total lipids in the human cutaneous epithelium was cholesterol and averaged about 1 per cent of the total weight of the skin.

Hess and Weinstock[4] have shown that the cholesterol (and ergosterol) contained in the skin can be activated by ultraviolet irradiations, giving it antirachitic properties. To one group of rats they fed irradiated skin, and to another group skin which had not been irradiated. The rats fed the irradiated skin were protected against rickets but the rats which had received the non-irradiated skin developed the vitamin deficiency.

Falkenheim[5] obtained similar results when he irradiated rats with ultraviolet and fed their skin to other rats.

Hou and Tso[6] found that human sebaceous cyst material has a high content of cholesterol and when fed to rats in the small amounts available is moderately antirachitic. To prove even

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[1]Hess, A. F., *J. Am. Med. Assoc.*, **84** (1925): 1910.

[2]Waddell, J., *J. Biol. Chem.*, **105** (1934): 711.

[3]Eckstein, H. C., and Wile, U. J., *J. Biol. Chem.*, **69** (1926): 181.

[4]Hess, A. F., and Weinstock, M., *J. Biol. Chem.*, **64** (1925): 181.

[5]Falkenheim, C., *Klin. Wochschr.*, **5** (1926): 2071.

[6]Hou, H. C., and Tso, A., *Chinese J. Physiol.*, **4** (1930): 93.

more definitely that materials of this kind can acquire antirachitic properties they cured severe rickets in rabbits by exposing the outer surface of the ears to ultraviolet light. When, however, this surface was cleansed with ether immediately before the exposure to the irradiations, only slight healing took place. The sebaceous secretion is believed to be responsible for the effects of the irradiation and easily penetrates the skin and produces its healing effect in the whole system.

An editorial in the Journal of the American Medical Association[7] seems to deny that a sufficient amount of vitamin D can be absorbed through the skin for any beneficial effects, and its loss from the skin surface or its reapplication after washing is of no consequence to health. The article, however, supports its claims with no scientific data.

The findings of the authors in previous experiments,[8] on the topical application of vitamin D to rats, are conclusive evidence that this vitamin can be readily absorbed through the skin and in sufficient quantities to cure severe rickets.

In the present experiments the authors were concerned only with determining whether washing removes such necessary fractions from the human skin. It is a well known fact that in the process of washing, an oily fraction is removed. McSwiney[9] has made a comprehensive study of the composition of human perspiration. The authors' experiments were conducted to determine whether this oily substance contained vitamin D or its precursor.

EXPERIMENTAL

First Experiment—An oily fraction was obtained from the skin of 23 male students of high school age who had spent

[7] *J. Am. Med. Assoc.*, **109** (1937): 509.

[8] Helmer, A. C., and Jansen, C. H., *this publication*, **1** (1937): 83.

[9] McSwiney, B. A., *Proc. Roy. Soc. Med.* **27** (1934): 839.

2 hr. exercising in an indoor gymnasium. The method of collecting this fraction was as follows: The young men stripped to the waist and washed with pieces of clean, sterile terry cloth, moistened with clear water. The cloths had been carefully extracted with ether in order to make certain that the cloths themselves contained no vitamin D precursor. After this washing, the men dried themselves with similar pieces of clean, sterile cloth. All the cloths and water were collected. The cloths were extracted three times with clear water and once with ether, and the combined water from all the washings was extracted three times with ether. The total ether extract was evaporated to a volume of 50 cc.

This washing of the upper torso was a mere preliminary to the boys' usual shower taken after exercise and the fraction of "skin oil" obtained in the extraction process would therefore probably be but a small per cent of what is lost from the body in the case of their usual hot bath.

The ether concentrate was irradiated in quartz test tubes for 1 hr., the source of irradiation being a quartz mercury arc from which all radiations shorter than approximately 2900 Å had been filtered.

Twenty rats, about 30 days old and weighing between 35 and 50 g., were prepared for this experiment. They were fed the rachitogenic diet specified in the United States Pharmacopoeia, Eleventh Decennial Revision, 1936.

The rats were weighed twice weekly during the 5-week depletion period, at the end of which time they were X-rayed to determine the degree of rickets. The radiographs were of the joint between the femur and tibia of the left hind leg.

The animals, placed in individual cages, were divided into two groups: one, the control, which continued to receive only the basal diet during the experimental period, and the other, the experimental, which received the basal diet plus the irradiated "skin oil" solution. The experimental rats were fed

the supplemented diet for six days and X-rays were taken of each animal on the ninth day.

The results are recorded in Table I and the radiographs are shown on Plates I to VII.

Eight rats showed a pronounced healing of rickets. One rat died and one was eliminated because of loss of weight during the test period. Of the 11 rats used as control none showed healing of rickets although 2 were eliminated because of loss of weight during the test period.

An average of 30 mg. of fat-soluble substance was removed from each boy by the method described, and an average of 7 mg. of this irradiated fat-soluble substance was fed daily to each of the 10 rats.

Second Experiment—The previous experiment gave ample proof that washing does remove the vitamin D precursor from the skin. This second experiment was conducted to see whether vitamin D itself, formed by irradiating the skin with ultraviolet light, is also removed by washing.

A group of 24 male students of high school age, just before going through their routine exercises in an indoor gymnasium, exposed their bodies to the radiations of a 500-watt quartz mercury arc for 10 min. at a distance of 6 ft. After playing for 2 hr. they washed with terry cloths and clear water as in the previous experiment. The water and cloths were extracted with ether, and the ether solution evaporated to a volume of 100 cc. This concentrate was added to the diet of the first group of experimental rats.

A second group of 24 students, of about the same age, were irradiated, as previously described, and after exercising, washed with terry cloths, soap and water. The soap used was ordinary toilet soap, containing no vitamin D. The method of washing was as follows: Lather was prepared with a moistened cloth and the body washed with it. A clean, damp cloth

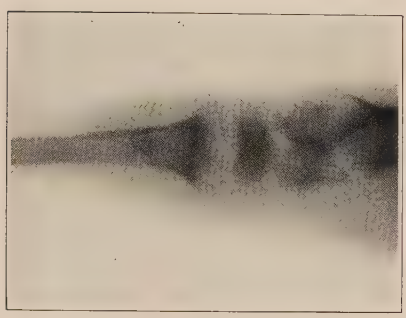
PLATE I

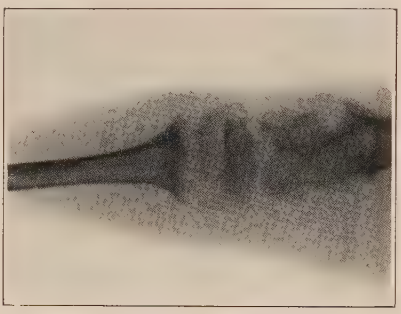
First Experiment

Radiographs showing the effect of feeding daily for 6 days, 7 mg. of irradiated fraction removed from human skin by washing.

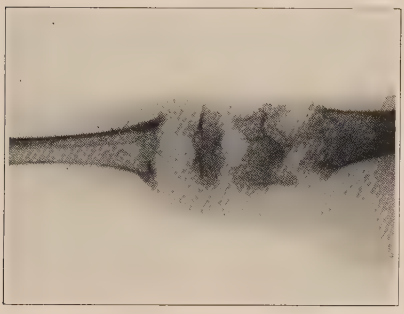
Before Treatment

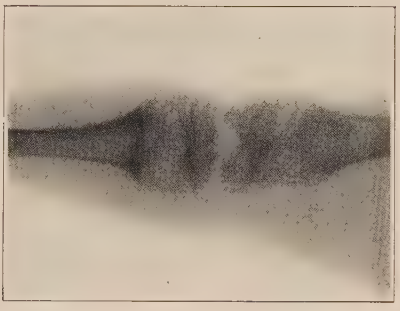
After Treatment



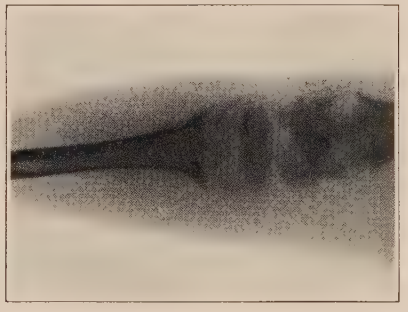


Rat No. 1





Rat No. 2



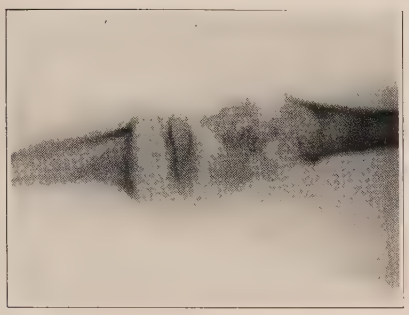
Rat No. 4

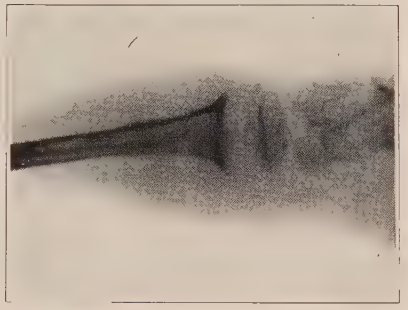
PLATE II

First Experiment—Continued

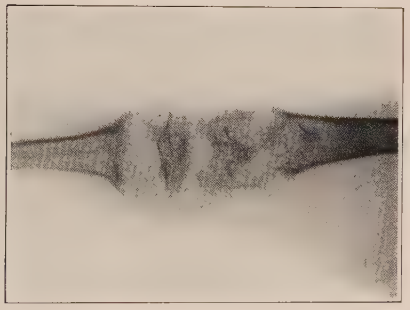
Radiographs showing the effect of feeding daily for 6 days, 7 mg. of irradiated fraction removed from human skin by washing.

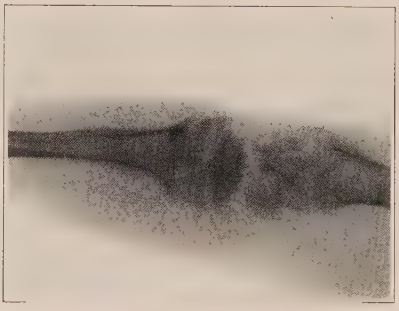
Before Treatment | After Treatment



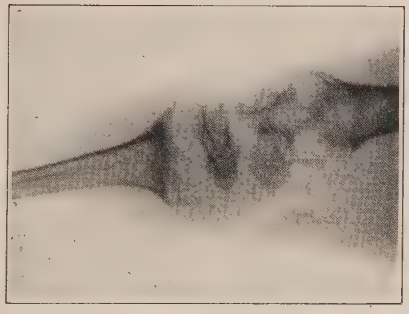


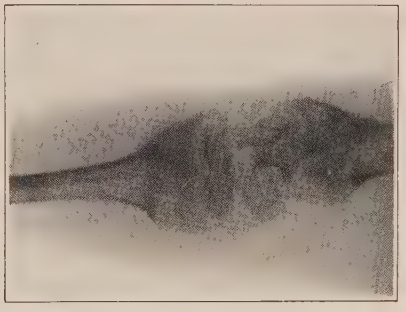
Rat No. 6





Rat No. 7





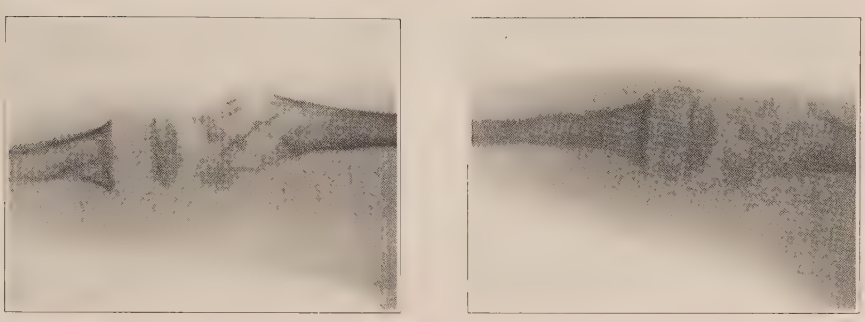
Rat No. 9

PLATE III

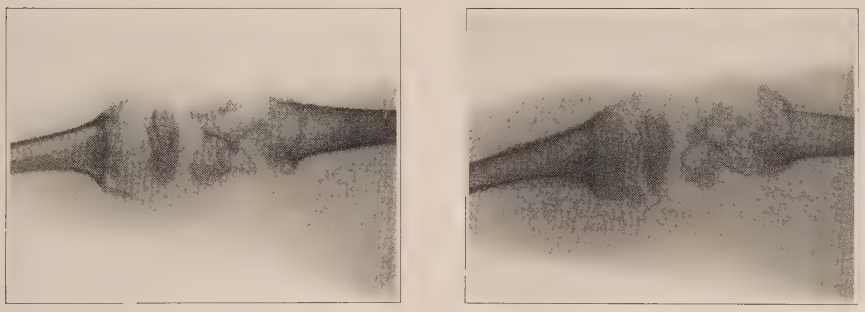
First Experiment—Continued

Radiographs showing the effect of feeding daily for 6 days, 7 mg. of irradiated fraction removed from human skin by washing.

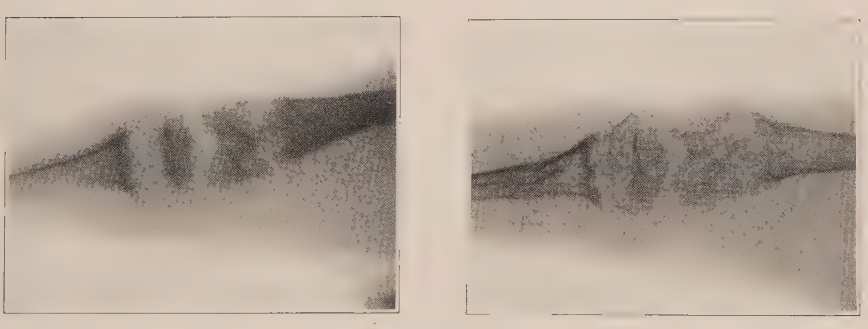
Before Treatment | After Treatment



Rat No. 12



Rat No. 13



Rat No. 14

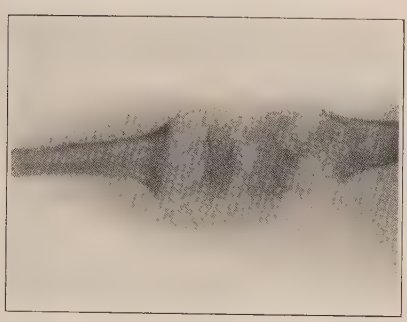
PLATE IV

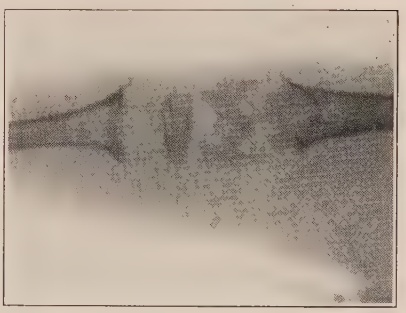
First Experiment—Continued

Radiographs of negative control rats.

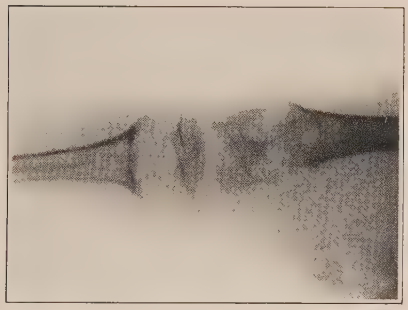
Beginning of Test Period

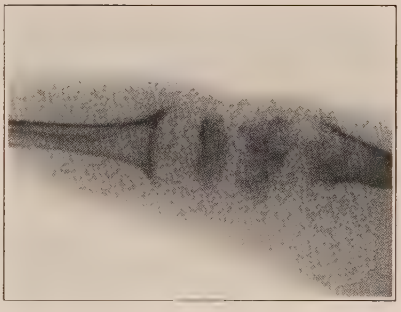
End of Test Period



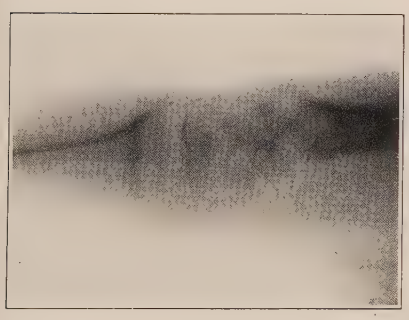


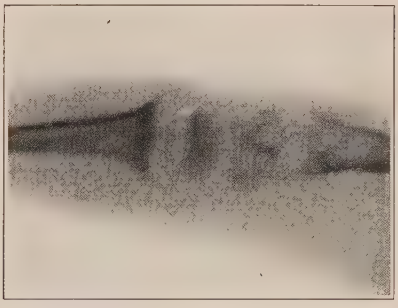
Rat No. 3





Rat No. 5





Rat No. 8

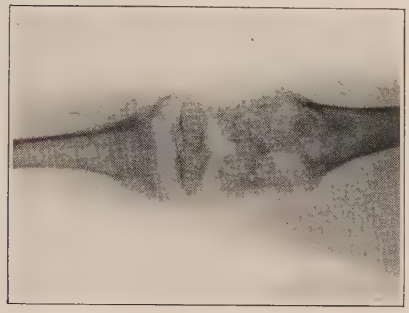
PLATE V

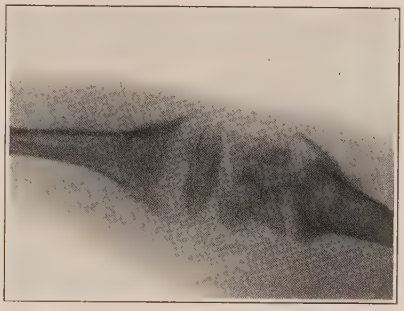
First Experiment—Continued

Radiographs of negative control rats.

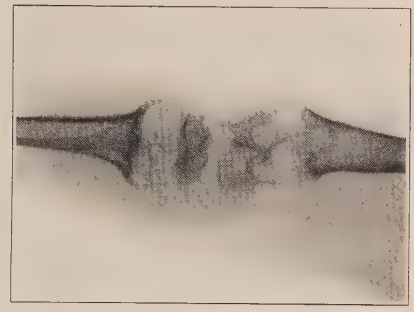
Beginning of Test Period

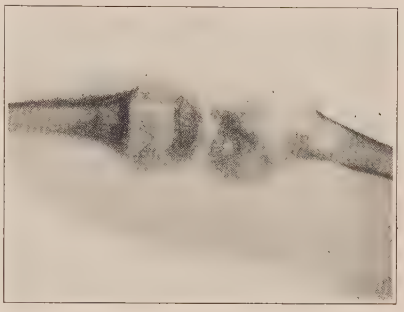
End of Test Period





Rat No. 9





Rat No. 10

Rat No. 11

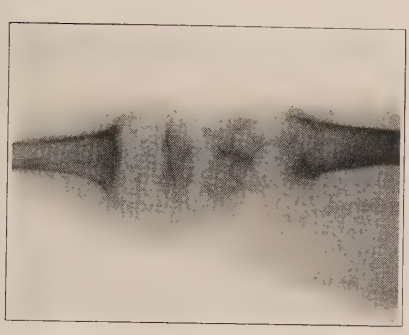
PLATE VI

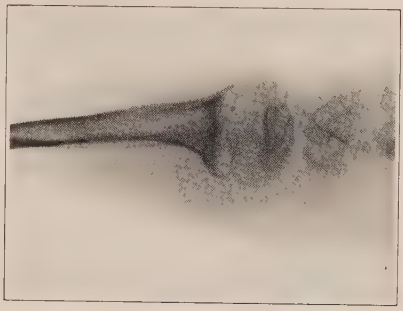
First Experiment—Continued

Radiographs of negative control rats.

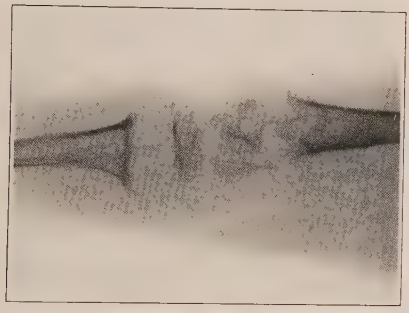
Beginning of Test Period

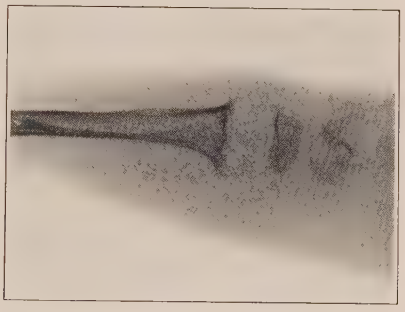
End of Test Period



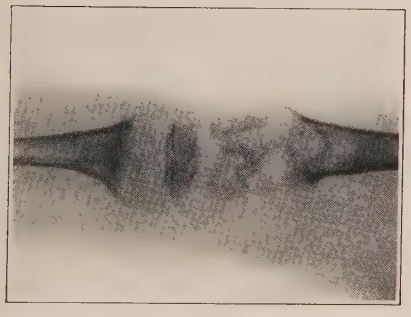


Rat No. 12





Rat No. 13



Rat No. 14

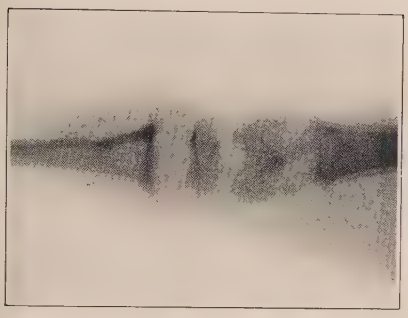
PLATE VII

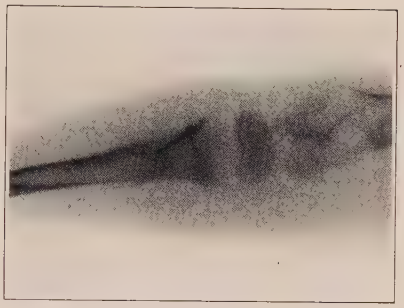
First Experiment—Continued

Radiographs of negative control rats.

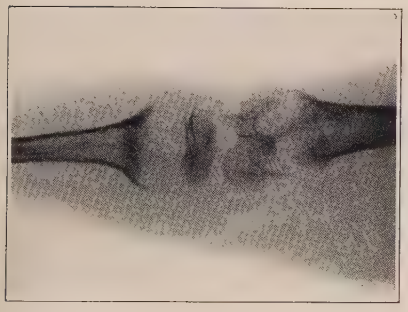
Beginning of Test Period

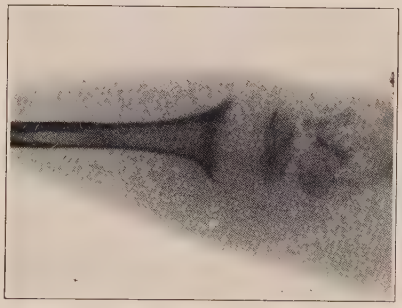
End of Test Period





Rat No. 15





Rat No. 16

X-Ray Diagnoses for Rickets

TABLE I

Group I—Experimental

Rats fed 7 mg. of irradiated "skin oil" daily for 6 days

	Degree of Rickets		Degree of Healing
	December 17	December 30	
Rat No. 1	Very severe	Severe	Pronounced
2	Very severe	Moderate	Pronounced
*4	Very severe	Severe	Pronounced
5	Very severe	Dead	
6	Very severe	Severe	Pronounced
7	Very severe	Mild	Pronounced
9	Very severe	Mild	Pronounced
12	Very severe	Moderate	Pronounced
13	Very severe	Moderate	Pronounced
14	Very severe	Moderate	Pronounced

Group II—Negative Control

	Degree of Rickets		Degree of Healing
	December 18	December 31	
Rat No. 3	Very severe	Very severe	No healing
5	Very severe	Very severe	No healing
*8	Severe	Severe	No healing
9	Severe	Severe	No healing
10	Severe	Severe	No healing
11	Very severe	Very severe	No healing
*12	Very severe	Severe	Slight healing
13	Very severe	Very severe	No healing
14	Very severe	Very severe	No healing
15	Very severe	Very severe	No healing
16	Very severe	Very severe	No healing

*Evidence questionable because of loss of weight during test period.

X-Ray Diagnoses for Rickets

TABLE II

Group I—Rats fed "skin oil" removed from irradiated skin by water wash only.

	Degree of Rickets	
	September 29	October 12
Rat No. 1	Severe	Moderate
2	Severe	Moderate
3	Very severe	Very severe
4	Very severe	Moderate
5	Severe	Slight
6	Severe	No rickets
7	Severe	Slight
8	Very severe	Severe
9	Very severe	Moderate
10	Severe	Moderate

Group II—Rats fed "skin oil" removed from irradiated skin by water and soap wash.

	Degree of Rickets	
	September 29	October 12
Rat No. 1	Severe	Moderate
2	Very severe	Moderate
3	Very severe	Moderate
4	Very severe	Slight
5	Severe	Slight
6	Very severe	Moderate
7	Severe	Moderate
8	Severe	Slight
9	Very severe	Slight
10	Very severe	Slight
11	Very severe	Moderate

X-Ray Diagnoses for Rickets

TABLE II—Continued

Group III—Rats fed "skin oil" removed from non-irradiated skin by water wash only.

	Degree of Rickets	
	September 29	October 12
Rat No. 1	Very severe	Severe
2	Very severe	Very severe
3	Severe	Very severe
4	Severe	Moderate
5	Severe	Moderate
6	Very severe	Very severe
7	Severe	Slight
8	Severe	Very severe
9	Very severe	Severe
10	Severe	Moderate

Group IV — Negative Control

	Degree of Rickets	
	September 29	October 12
Rat No. 1	Very severe	Very severe
2	Very severe	Very severe
3	Moderate	Severe
4	Very severe	Very severe
5	Very severe	Severe
6	Severe	Severe
7	Very severe	Very severe
8	Severe	Very severe
9	Very severe	Very severe
10	Very severe	Very severe

was used to wipe the skin and a third cloth to dry it. These cloths were all collected and extracted three times with ether. This ether extract was filtered and concentrated to 100 cc. and was fed to the second group of experimental rats.

A third group of 24 students were not irradiated before exercising in the gymnasium for 2 hr. Immediately after the exercise they washed with clean cloths and clear water. The cloths and water were extracted as in the other groups, and the extract, after concentration, was irradiated in quartz test tubes for 20 min., at a distance of 20 in. from a 500-watt quartz mercury arc. This irradiated fat-soluble fraction was fed to the third group of rats.

The 41 rats used in this experiment were divided into four groups. Groups I, III and IV comprised 10 rats each, while Group II contained 11 rats. All the animals were fed a vitamin-D-free diet for 3 weeks and then X-rays were taken to determine the degree of rickets. Thereafter the rats in Group I were daily fed 1 cc. of the "skin oil" concentrate obtained from the first group of students. The ether was allowed to evaporate from the concentrate after it was poured over the daily ration of rat food. This feeding was continued for 8 days, and 3 days later X-rays were again taken to diagnose the rachitic condition of the animals.

The oil concentrate obtained from the second group of students was fed in the same manner to the rats of Group II, and the concentrate from the third group of boys was given to the rats of Group III, while the rats of Group IV were used as negative control, receiving the same ration as the other rats except that it contained no vitamin supplement.

The results of the experiment are recorded in Table II and typical radiographs are shown on Plates VIII to XI.

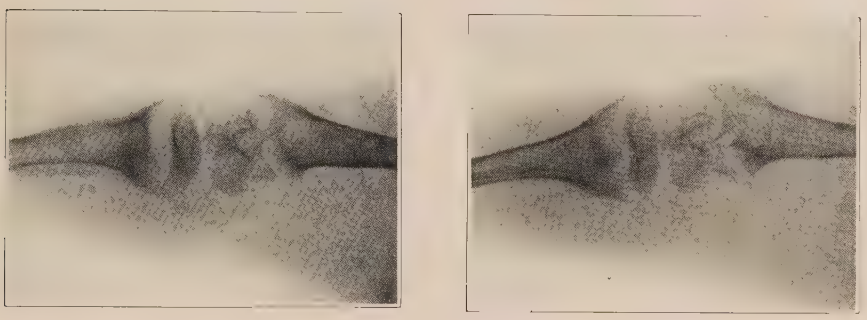
PLATE VIII

SECOND EXPERIMENT—GROUP I

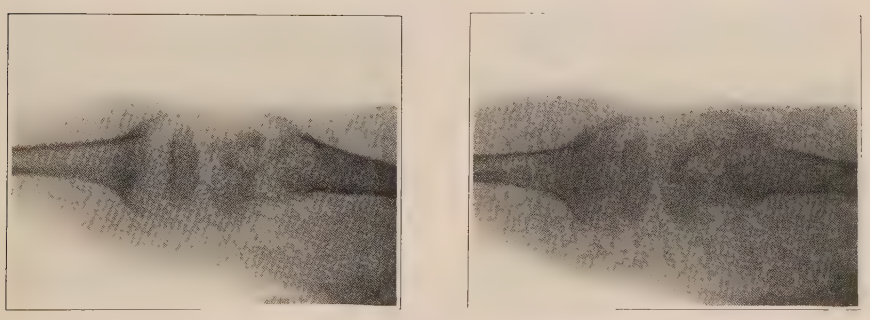
Typical radiographs of rats fed fraction removed from irradiated human skin by water wash only.

Before Treatment

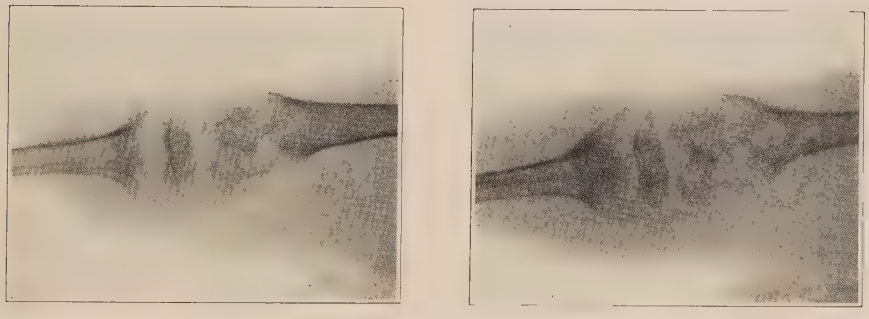
After Treatment



Rat No. 2



Rat No. 5



Rat No. 7

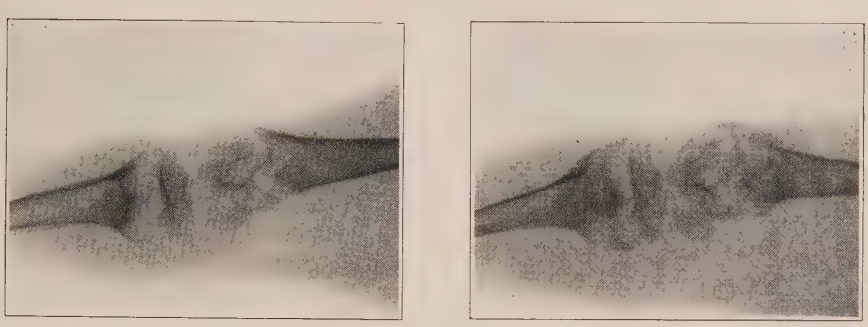
PLATE IX

SECOND EXPERIMENT—GROUP II

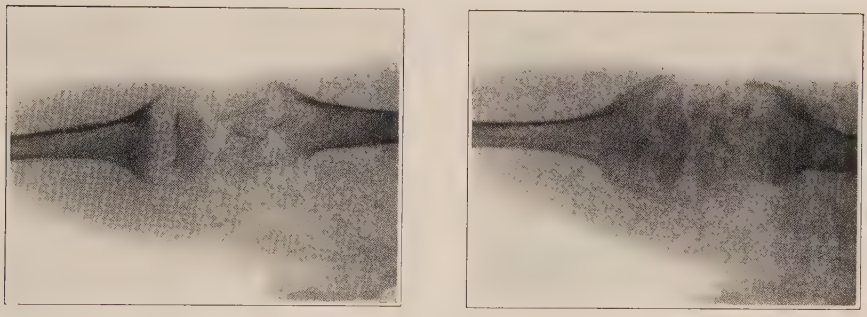
Typical radiographs of rats fed fraction removed from irradiated human skin by soap and water wash.

Before Treatment

After Treatment



Rat No. 1



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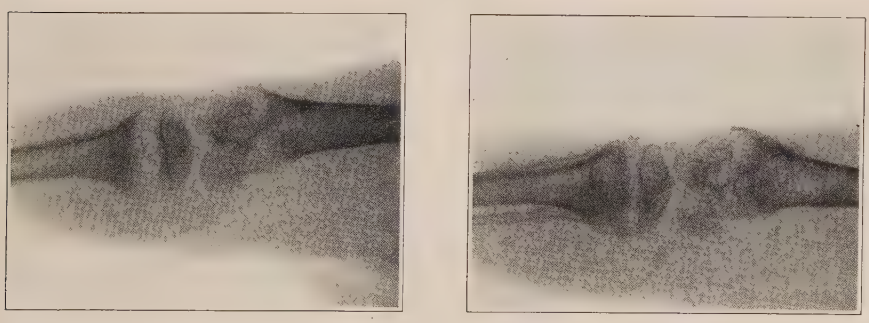


Rat No. 5

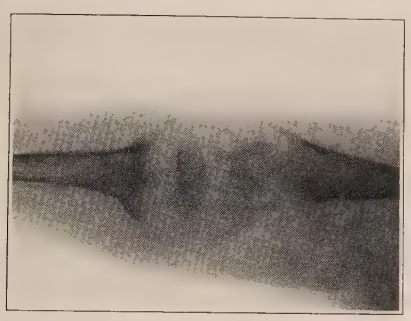
PLATE X

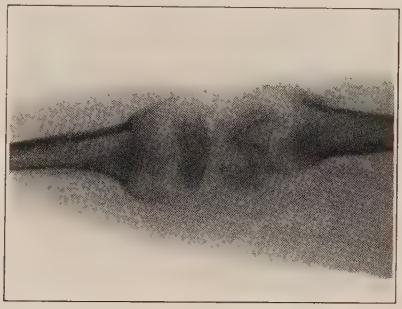
Second Experiment—Group III

Typical radiographs of rats fed fraction removed from non-irradiated human skin by water wash, but irradiated after extraction.

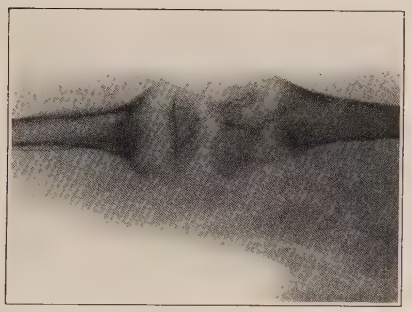
Before Treatment

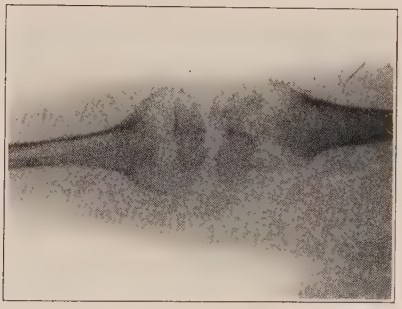
After Treatment



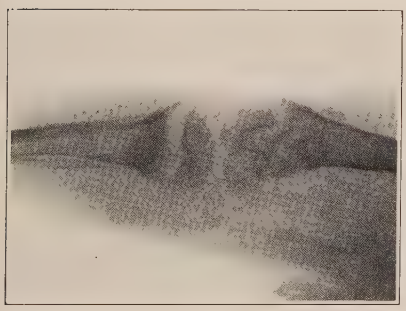


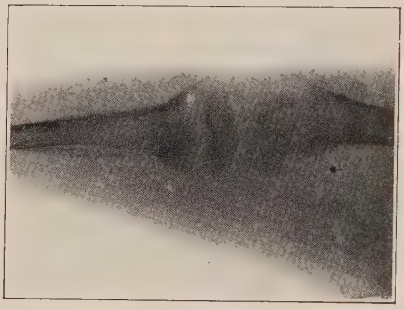
Rat No. 1





Rat No. 5





Rat No. 7

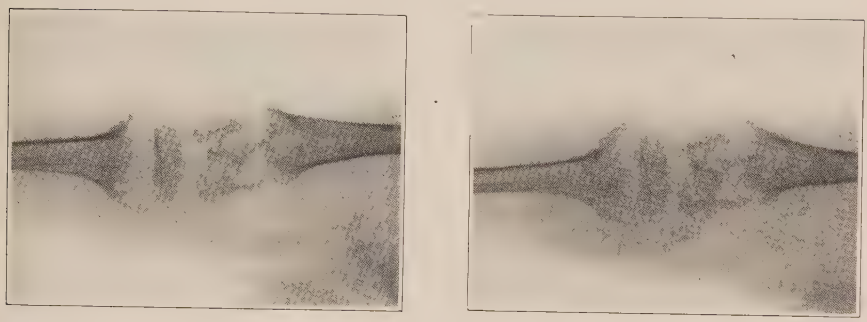
PLATE XI

SECOND EXPERIMENT—GROUP IV

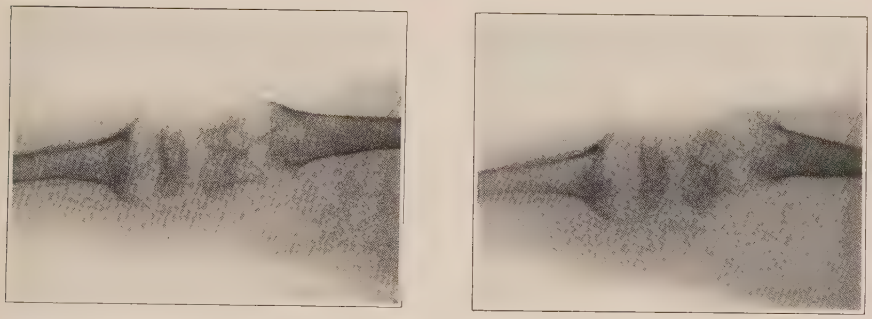
Typical radiographs of negative control rats.

Beginning of Test Period

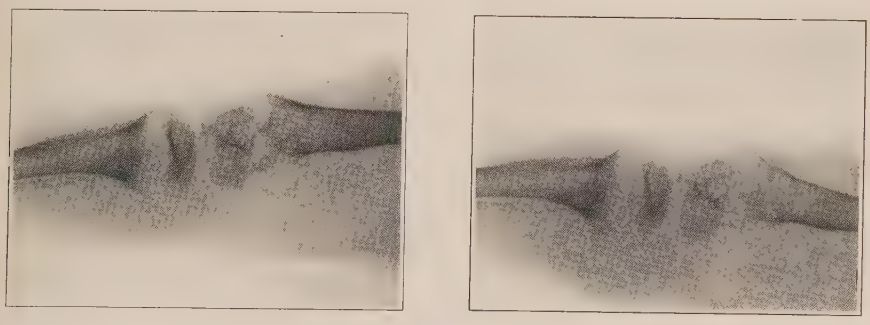
End of Test Period



Rat No. 5



Rat No. 7



Rat No. 8

Ninety per cent of the rats in Group I, which had been fed the "skin oil" removed from irradiated skin by means of a water wash only, showed definite healing of rickets ranging from slight to complete cures.

All the rats in Group II, which had been fed the "skin oil" removed from irradiated skin by water and soap wash, showed definite healing of rickets, ranging from slight to almost complete cures.

Among the rats to which the "skin oil" removed from non-irradiated skin by water wash only had been fed (Group III), healing of rickets occurred in but 60 per cent of the cases, and in the majority of cases to merely a slight degree.

During this same test interval the negative control rats became more severely rachitic.

CONCLUSIONS

1. Washing does remove fractions from the human skin, which ultraviolet rays can convert into antirachitic agents.

2. When the human skin is irradiated with ultraviolet light, vitamin D is formed in certain fractions of or upon the outer layer of skin, which washing with clear water will remove.

3. The activatable factor in or upon the outer layer of human skin is a precursor of vitamin D, for when the skin is not irradiated, the antirachitic properties are very much lessened.

4. The evidence presented in the two groups of experiments indicates that washing the human skin by the usual methods removes vitamin D and its precursors from the outer layer of skin.

5. There is definite evidence that the secretions from the skin contain precursors of vitamin D, which after irradiation

are to be reabsorbed by the body, and the removal of which tends to produce a dearth of the vitamin unless it be supplied in some other form.

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THE EFFECT OF LIGHT AND ATMOSPHERE ON THE DEVELOPMENT OF PLANT PIGMENTS

WILLIAM A. BECK, S.M.*

It is well known that in the higher plants chlorophyll can develop only in the presence of light.[1] Xanthophyll and carotene, however, can develop in the absence of light[2] but in the case of carotene, development takes place very slowly if the plant is not exposed to light. There seem to be no references in the literature to the development of these pigments as functions of pressure. It is the purpose of this investigation to determine the influence of light and pressure on the development of plant pigments in air, carbon dioxide and oxygen.

EXPERIMENTAL

The plants were exposed to the various atmospheres in a tank made of heavy metal. It had a thick piece of shatterproof glass as a top cover, which could be bolted firmly. Rubber gaskets made the top air-tight. The gas was admitted at the bottom and could be released at the top. This made it easy to eject the air and be sure the desired gas filled the chamber. The prevailing pressure was indicated on a gauge mounted on the top of the tank (Fig. 1). The interior of the tank was painted with several coats of enamel to insure abundant reflection of light so that every part of the plant would be illuminated.

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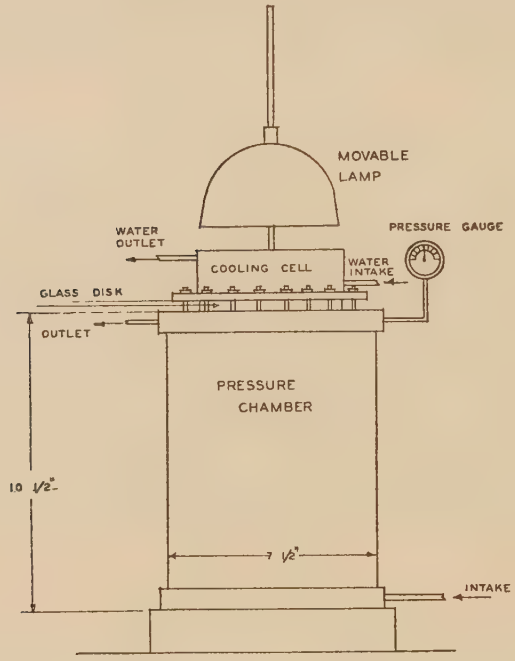
*Lecturing Professor, University of Dayton, Dayton, Ohio.

[1]Benecke-Jost, *Pflanzenphysiol.*, 1 (1924): 209; Heinricher, E., *Ber. Bot. Ges.*, 1 (1883): 433; Schimper, A. F., *Jahrb. Wiss. Bot.*, 16 (1885): 1246; Schmidt, A., *Beitr. z. Biol.*, 12 (1914): 269.

[2]Beck, W. A., *this publication*, 1 (1937): 109.

FIG. 1—*Device for Irradiating Plants under Pressure*



A cooling cell was mounted above the glass through which water was kept flowing while the experiment was in progress. Above the cooling tank a filament lamp was suspended in such a manner that the intensity of the illumination could be varied by raising or lowering the lamp. After a number of preliminary experiments made it evident which intensity was most desirable, the lamp was fixed in position so that the same illumination would prevail in all experiments. Moist German peat was kept in the bottom of the tank. One hundred per cent relative humidity was maintained during all the experiments.

Preliminary Experiments with Hubbard Squash Seedlings

In these first experiments squash seedlings were used. In some cases the plants were exposed in the tank for a number

of hours in air at normal pressure, and in others at excess pressure. The lamp was so arranged that, with the glass in place, 100 foot-candles were registered at the point where the plants stood. The surprising result was that no chlorophyll developed during the period of exposure. In diffused daylight, plants of the same age and kind, raised in the same manner, developed chlorophyll in a short time in the tank. There was no doubt that the glass absorbed certain wavelengths which were favorable for the development of chlorophyll. Sayre[3] showed that the effective radiant energy in developing chlorophyll increases with the wavelength to about 6800 Å and then ends abruptly. The fact that the glass in the head of the tank absorbed radiations must be borne in mind while interpreting the results of the following experiments. The glass did not, however, absorb all of the effective radiations for chlorophyll developed when the intensity of the light was increased. Little difference was found between the test plants and the control plants in these experiments. Some of the test plants were exposed in air at ordinary pressure and also at 27 lb. excess pressure; others in oxygen at 27 lb.; and still others in carbon dioxide at the same pressure. When the intensity of the light or the time of exposure was increased (without increasing the temperature, which varied little from 20° C.) the effect became evident in the general appearance of the cotyledons.

Experiments with Hubbard Squash

Hubbard squash seedlings were exposed in the tank, in the manner described, for a period of 22 hr. in atmospheres of air, carbon dioxide and oxygen, which were under a pressure of 24 lb. in excess of normal atmospheric pressure.

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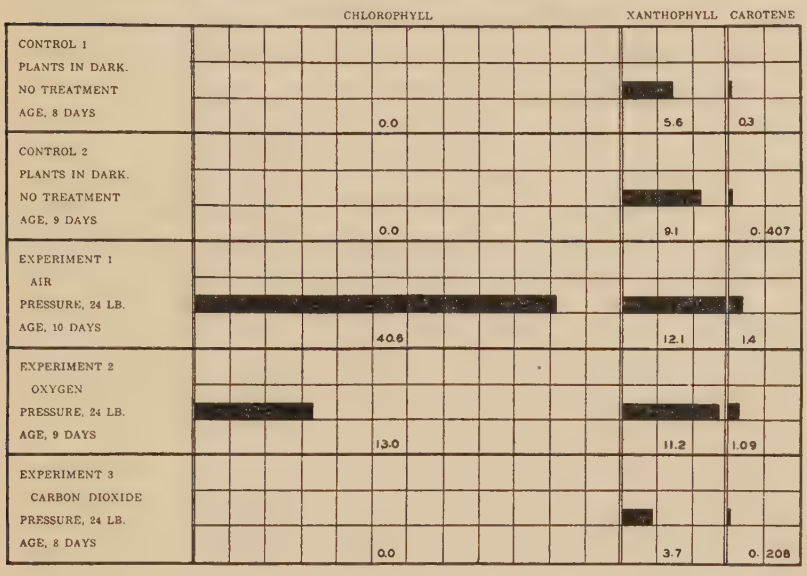
[3]Sayre, J. T., *Plant Physiol.*, 3 (1928): 71.

TABLE I

The Amount of Pigment Developed During 22 hr. in Hubbard Squash Seedlings in Atmospheres of Compressed Air, Oxygen and Carbon Dioxide

Factors	Mg. per 100 g. Wet Weight			Mg. per 10 g. Pulp			Mg. per 1000 Cotyledons			Quotients		
	Chlorophyll	Xanthophyll	Carotene	Chlorophyll	Xanthophyll	Carotene	Chlorophyll	Xanthophyll	Carotene	Ch/X	Ch/Car	X/Car
Control 1 Plants in dark. No treatment Age, 8 days	0.0	5.7	0.3	0.0	4.8	2.5	0.0	5.6	0.3	0.0	0.0	18.3
Control 2 Plants in dark. No treatment Age, 9 days	0.0	8.9	0.41	0.0	8.17	0.37	0.0	9.1	0.407	0.0	0.0	21.7
Experiment 1 Air Pressure, 24 lb. Age, 10 days	35.4	10.5	1.3	47.4	14.1	1.7	40.6	12.1	1.4	3.33	27.7	8.3
Experiment 2 Oxygen Pressure, 24 lb. Age, 9 days	10.2	9.9	0.8	11.0	9.7	0.947	13.0	11.2	1.09	1.1	11.9	10.3
Experiment 3 Carbon Dioxide Pressure, 24 lb. Age, 8 days	0.0	4.1	0.23	0.0	4.04	0.23	0.0	3.7	0.208	0.0	0.0	17.7

FIG. 2—*The Amount of Pigment Developed During 22 hr. in Hubbard Squash Seedlings in Atmospheres of Compressed Air, Oxygen and Carbon Dioxide*



Control 1—Some of the seedlings were taken from the dark chamber when they were 8 days old and the pigment of the cotyledons extracted and determined quantitatively. The values are given in Table I and are graphically represented in Fig. 2. They serve as a basis of comparison for the seedlings treated in the tank.

Control 2—Other seedlings were taken from the sprouting chamber when they were 9 days old and the contents of the cotyledons determined. The results are likewise given in Table I.

Experiment 1—Compressed Air—In the first experiment the seedlings were taken from the dark chamber when they were 9 days old and exposed in the tank for 22 hr., in an atmosphere of compressed air, after which the amounts of the pigments

were determined. The final age was approximately 10 days. The results are given in Table I.

Experiment 2—Compressed Oxygen—This experiment was similar to the previous one except that oxygen at 24 lb. in excess of atmospheric pressure was used instead of compressed air. The seedlings were 9 days old. The results are given in Table I.

Experiment 3—Compressed Carbon Dioxide—In this experiment the atmosphere was carbon dioxide at 24 lb. excess pressure. The results are given in Table I. The development was very poor in this last experiment, the plants being limp when removed, which was not the case in the other experiments. Since all conditions were the same as those in the preceding experiments, with the exception of the atmosphere of carbon dioxide, it seems evident that CO_2 inhibits the general metabolism. No chlorophyll was present but in the process of saponification, instead of chlorophylin, a yellow liquid appeared which, when treated with ether, left the ether clear. The "brown phase" was never passed. This solution was similar to the one which appears when the etiolated cotyledons are taken directly from the sprouting chamber and the pigments extracted. If this product is protochlorophyll or leukophyll, then it would appear that the material neither broke down nor developed into chlorophyll. There can be no question of flavones since all flavones were previously removed. There was less xanthophyll present than in the control, as was also the case with carotene. Not only did carotinoid pigment fail to develop but some appears to have disintegrated during the treatment.

There was some development of pigment in the atmosphere of oxygen, but the plants appeared to be less vigorous than plants exposed in air at normal pressure. Some chlorophyll developed. The regions in which it did develop were not evenly distributed over the cotyledons. Cotyledons usually begin to

get green on the edges, but in this case the green regions first appeared in the middle of the lamina in spite of the fact that the light played evenly over them. The edges were still yellow at the conclusion of the experiment. An appreciably greater amount of xanthophyll developed, in fact, almost as much as in the case where compressed air was used (Table I). The same was true for carotene. This emphasizes what other investigators have already noted: there appears to be no relation between the development of chlorophyll and the carotinoids. There is difference of opinion regarding a possible relation between xanthophyll and carotene but the results of this experiment do not justify a judgment one way or the other, although it may be noted that the amount of carotene developed in the carbon dioxide was very much less than that developed in every other instance.

The best development of all pigments occurred when compressed air was employed. All of the pigments developed in appreciable quantities. Not only did the compressed air fail to inhibit the general activity of the plant but actually appeared to stimulate it.

It may therefore be concluded that the carotinoids developed well in compressed air but not in compressed oxygen or compressed carbon dioxide. Chlorophyll failed to develop in carbon dioxide and very little was formed in oxygen. The development of pigment was roughly proportional to the general metabolism of the plants as judged by the tone of the plants.

Preliminary Experiments with Sunflower Seedlings

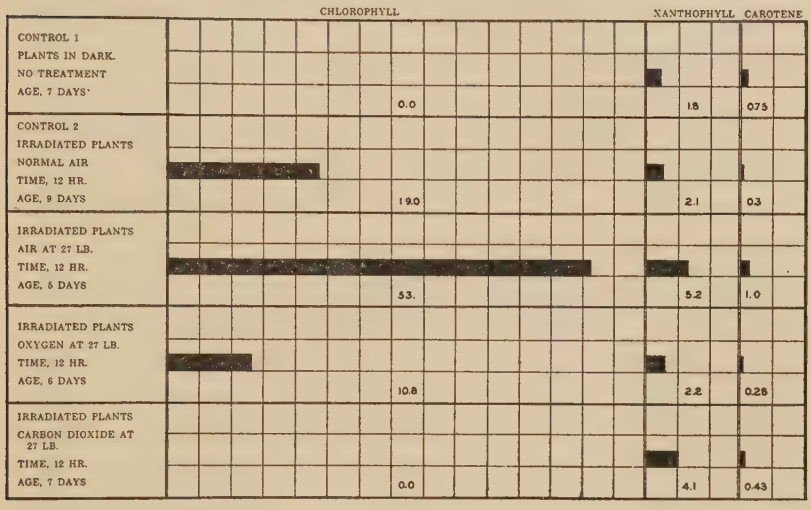
The intensity of the illumination was increased to such an extent that chlorophyll became evident in the cotyledons in a short time. The temperature during the experiments was again approximately 20° C. The time of exposure was 12 hr. for all experiments. The plants were not of the same age, but

TABLE II

The Amount of Pigment Developed in Sunflower Seedlings in Atmospheres of Normal and Compressed Air, Compressed Oxygen and Compressed Carbon Dioxide

Factors	Mg. per 100 g. Wet Weight			Mg. per 10 g. Pulp			Mg. per 1000 Cotyledons			Quotients		
	Chlorophyll	Xanthophyll	Carotene	Chlorophyll	Xanthophyll	Carotene	Chlorophyll	Xanthophyll	Carotene	Ch/X	Ch/Car	X/Car
Control 1 Plants in dark. No treatment Age, 7 days	0.0	3.3	1.4	0.0	3.4	1.4	0.0	1.8	0.75	0.0	0.0	2.4
Control 2 Irradiated Plants Normal Air Time, 12 hr. Age, 9 days	25.0	2.8	0.4	28.0	3.2	0.48	19.0	2.1	0.30	8.9	62.5	7.0
Irradiated Plants Air at 27 lb. Time, 12 hr. Age, 5 days	71.0	6.9	1.3	85.0	8.27	1.55	53.0	5.2	1.0	10.3	5.5	5.3
Irradiated Plants Oxygen at 27 lb. Time, 12 hr. Age, 6 days	16.1	3.3	0.41	17.5	3.58	0.45	10.8	2.2	0.28	4.9	39.2	8.17
Irradiated Plants Carbon Dioxide at 27 lb. Time, 12 hr. Age, 7 days	0.0	7.1	0.74	0.0	7.3	0.77	0.0	4.1	0.43	0.0	0.0	9.5

FIG. 3—*The Amount of Pigment Developed in Sunflower Seedlings in Atmospheres of Normal and Compressed Air, Compressed Oxygen and Compressed Carbon Dioxide*



all seeds were planted at the same time and the seedlings raised under the same conditions. The seed coats were removed just before each experiment was begun. The results are assembled in Table II and represented graphically in Fig. 3.

In the top row are given the results obtained from the control plants, which were taken directly from the sprouting chamber. In the second row are given the results obtained from plants exposed for 12 hr. in air at ordinary pressure. These two experiments may be considered the controls. In the next three rows are the results obtained when the atmosphere was air, oxygen and carbon dioxide respectively, all at excess pressure of 27 lb./sq. in. The amount of pigment is expressed as a function of the wet weight, of the pulp, and of the number of cotyledons. It was found that the last mode of expressing the increment is the best.

The greatest amount of chlorophyll developed in the plants exposed in compressed air for 12 hr. One factor which may

cast some doubt on the validity of the conclusions is, that plants, when kept too long in the dark, might not develop chlorophyll as readily as plants exposed to light. In the pressure experiments the plants were 5 days old, while the plants exposed in normal air were 9 days old. These doubts were raised by later experiments when it was found that unusually old sprouts were slow to develop chlorophyll. Assuming the existence of chlorophylogen, it is possible that this substance would deteriorate if too great an interval of time elapsed before irradiation was begun.

It is interesting, however, that these results agree with the previous ones which indicated that compressed air stimulated the production of pigment and likewise the general metabolism. No chlorophyll developed in the plants exposed in carbon dioxide. The color of the cotyledons had not changed greatly and their general appearance showed them to be deteriorating. It seemed possible that a longer exposure might cause chlorophyll to develop. Less chlorophyll developed in the plants exposed in oxygen than in those exposed in air. It appeared evident that the compressed air distinctly stimulated the development of chlorophyll. What the mechanism of the increased development might be is difficult to state. It seemed likely that the increased pressure would cause more air to penetrate the plant and aid the exchange of gases necessary for the development of the pigment.

Most xanthophyll developed in the plants exposed in carbon dioxide. This was quite unexpected and is difficult to explain. Least xanthophyll developed in the plants exposed in air at atmospheric pressure, and but little more in the plants exposed in oxygen. If it be assumed that decreased metabolism occurs in the order, air, oxygen, carbon dioxide, then it may be stated that the development of xanthophyll increased with the decrement of metabolism. Compressed air, however, stimulated the formation of xanthophyll.

The production of carotene was slight in all cases. It was least in the plants exposed in oxygen and most in the plants exposed in compressed air.

All the pigments showed an increment in the plants exposed in compressed air over those exposed in normal air. The plants exposed in compressed air were vigorous and showed a tendency to form roots. Upon removal from the abnormal condition they continued to thrive on the laboratory table for many hours, which indicated that they did not suffer seriously from the exposure.

It is possible that the suction tension of the cells varied under the unusual external pressure which in turn would cause the amount of water intake to increase. There is no direct evidence that such an increased suction tension actually prevailed. The wet weight per cotyledon was 0.074 g. for the plants in compressed air, while it was 0.0738 g. for the plants in normal air. The difference is not sufficiently great to allow a definite conclusion. The experimental plants taken directly from the dark had a wet weight of 0.055 g. per cotyledon and those exposed in carbon dioxide 0.057 g. per cotyledon. These figures agree with the others in indicating a low metabolism in the plants not exposed in normal or compressed air.

The study of the pulp led to the same conclusion.

The increment in chlorophyll is in keeping with the general development of the plant. It developed in even greater quantities in the plants exposed in compressed air than might have been expected from the general increase in metabolism induced by the compressed air. The xanthophyll increment was greatest in the plants exposed in carbon dioxide. This was unexpected and is difficult to explain.

Second Series of Experiments with Sunflower Seedlings

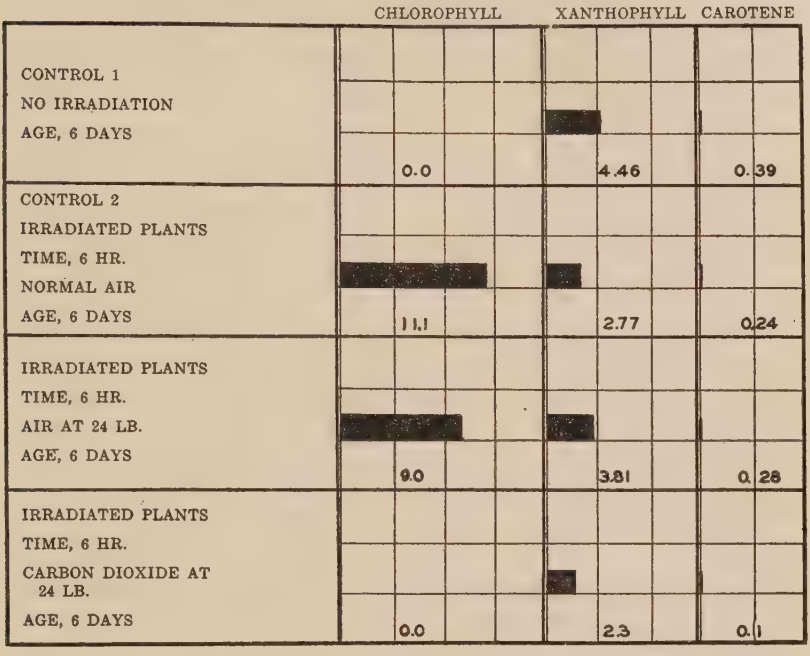
In the following experiments seedlings of sunflower (*helianthus annuus*) were employed. The results of the experiments

TABLE III

Amount of Pigment Developed in Sunflower Seedlings During 6 hr. Exposure

Factors	Mg. per 100 g. Wet Weight			Mg. per 10 g. Pulp			Mg. per 1000 Cotyledons			Quotients		
	Chloro-phyll	Xantho-phyll	Caro-tene	Chloro-phyll	Xantho-phyll	Caro-tene	Chloro-phyll	Xantho-phyll	Caro-tene	Ch/X	Ch/Car	X/Car
Control 1 No irradiation Age, 6 days	0.0	6.3	0.55	0.0	10.0	0.8	0.0	4.46	0.39	0.0	0.0	11.3
Control 2 Irradiated Plants Time, 6 hr. Normal Air Age, 6 days	18.4	4.7	0.4	17.1	4.25	0.377	11.1	2.77	0.24	4.0	45.7	11.3
Irradiated Plants Time, 6 hr. Air at 24 lb. Age, 6 days	14.0	5.9	0.44	18.5	7.8	0.58	9.0	3.81	0.28	2.37	31.68	13.3
Irradiated Plants Time, 6 hr. Carbon Dioxide at 24 lb. Age, 6 days	0.0	3.3	0.26	0.0	3.71	0.29	0.0	2.3	0.1	0.0	0.0	12.6

FIG. 4—*Amount of Pigment Developed in Sunflower Seedlings During 6 hr. Exposure*



were constantly checked with control plants grown under similar conditions save for the particular factors that were being studied. The experimental and the control plants were of the same age except when otherwise indicated. Only large and solid seeds were selected so that the cotyledons might be approximately the same size. This was of some importance since the best way to estimate the development of pigment was as a function of the number of cotyledons.

First Group

In this series the plants were introduced into the tank for treatment when they were 6 days old. The results are given in Table III and the graphical representation of the development is given in Fig. 4.

In the first control experiment the cotyledons were taken from plants growing in the dark chamber under normal conditions. In the second control experiment the plants received the same illumination as the experimental plants but under normal atmospheric pressure while the experimental plants were subjected to a pressure of 24 lb. in excess of atmospheric pressure. One set of experimental plants was in an atmosphere of compressed air, another in an atmosphere of compressed carbon dioxide, and the final set in an atmosphere of compressed oxygen. The period of treatment for all three sets and the second control experiment was 6 hr. The amount of pigment was expressed as a function of the wet weight, as a function of the pulp, and as a function of the number of cotyledons.

The results again showed that light is essential for the development of the chlorophyll, though xanthophyll and carotene were present before the plants were irradiated. Chlorophyll failed to develop in the carbon dioxide atmosphere. It seemed possible that some might develop if the time of irradiation were extended. Chlorophyll developed well in the compressed air but not as well as in air at normal pressure. While compressed air does not prevent the development of chlorophyll it does seem to decrease the rate of formation. (In later experiments, when the plants were exposed for a longer time, there was greater development). It could not be the lack of oxygen as in the case of the inhibition produced by the carbon dioxide. The greater pressure of the air would tend to carry the gases into the deeper inter-cellular spaces, which would probably aid their ready diffusion through the cell walls. It would be difficult to say just how much time would be necessary for the possible increase in the rate to manifest itself. The development of pigment should at least be as great as for the normal plants. Even though this supposition is correct, it would be safe to say that 6 hr. would be

insufficient to produce the evidence under the given conditions. With increased irradiation, however, this time might be sufficient, for the development of the pigment no doubt is dependent on many factors, e.g., the irradiation, the metabolism and the rate of diffusion of gases to and from the protoplasm.

It is difficult to see any relation between the development of the chlorophyll and the carotinoid pigments. A smaller amount of each carotinoid pigment was found in the plants exposed in the compressed carbon dioxide atmosphere than was present before the exposure was made. At the end of the experiment there were only 2.3 mg. of xanthophyll per 1000 cotyledons, while in the first control group, which was the same age, the plants contained nearly twice as many milligrams (4.46); the carotene content was nearly four times as great in the first control as in the experimental plants (0.39/0.1). These results tend to strengthen the supposition that carbon dioxide inhibits the general metabolism.

It is interesting, if not significant, that both xanthophyll and carotene are present in greater quantities in the plants exposed in compressed air than in those exposed in air at normal pressure, but in both cases the amount was less than in the non-irradiated plants of the same age. (Table III and Fig. 4). One is reminded of the findings of Norris[4] that carotene suffered a definite decrease when the seedlings were irradiated and only later, after an initial depression, did it increase steadily. A new difficulty arises when the present results are compared with those of Norris. In his case the effect was noted in less than 2 hr. and after 5 hr. the total amount was already greater than the initial value, while in the present case the initial amount had not yet been reached after 6 hr. It must be remembered that Norris did not interpose a glass

[4]Norris, R. J., *Bull. Basic Sci. Research*, 5 (1933): 23.

between the source of light and the exposed plants while in the author's experiment a thick glass was used, which absorbed much effective radiation.

Second Group

From the previous experiments it appears that the unusual pressure does not seriously injure the plants and does not prevent the development of the plant pigments. It may, however, depress the metabolism.

An experiment was carried out in which 4-day-old plants were continuously irradiated in the usual manner in the tank for 45 hr., the atmosphere being air at 24 lb. excess pressure. Three control experiments were conducted.

In the first, the plants were taken directly from the dark chamber in which they were sprouted and no treatment was given. These values served to check the initial condition of the experimental plants which were taken from the same lot and placed in the tank at the time the plants for the first control were analyzed.

The second group of control plants was taken from the same lot when the exposure of the experimental plants was concluded.

The plants for the third control experiment were of the same final age as the experimental plants, but had been planted later, though from the same crop of seeds. The time of exposure was 6 hr. instead of 45 hr.; otherwise the conditions were the same. The results are given in Table IV and are represented graphically in Fig. 5.

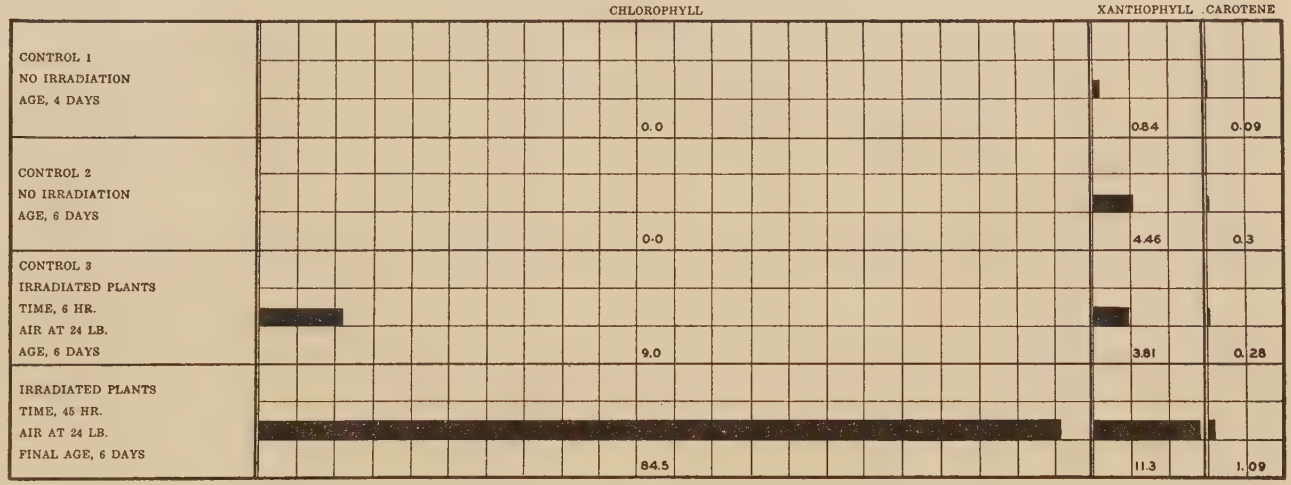
At the end of the 45-hour period the experimental plants were vigorous, erect and turgescent. They were even more turgescent than when placed in the tank, and more than the plants of the same age still in the dark chamber, which were not even erect. In interpreting this condition phototropism

TABLE IV

The Development of Plant Pigments in Sunflower Seedlings Under Compressed Air During Periods of 6 hr. and 45 hr.

Factors	Mg. per 100 g. Wet Weight			Mg. per 10 g. Pulp			Mg. per 1000 Cotyledons			Quotients		
	Chloro-phyll	Xantho-phyll	Caro-tene	Chloro-phyll	Xantho-phyll	Caro-tene	Chloro-phyll	Xantho-phyll	Caro-tene	Ch/X	Ch/Car	X/Car
Control 1 No irradiation Age, 4 days	0.0	1.5	0.17	0.0	1.04	0.11	0.0	0.84	0.09	0.0	0.0	9.0
Control 2 No irradiation Age, 6 days	0.0	6.3	0.55	0.0	10.0	0.88	0.0	4.46	0.3	0.0	0.0	11.3
Control 3 Irradiated Plants Time, 6 hr. Air at 24 lb. Age, 6 days	14.0	5.9	0.44	18.5	7.8	0.58	9.0	3.81	0.28	2.37	31.68	13.3
Irradiated Plants Time, 45 hr. Air at 24 lb. Final age, 6 days	108.0	14.5	1.5	130.7	17.8	1.75	84.5	11.3	1.09	7.48	77.5	10.0

FIG. 5—*The Development of Plant Pigments in Sunflower Seedlings Under Compressed Air During Periods of 6 hr. and 45 hr.*



must be taken into consideration but phototropism alone would not explain their vigorous state as is evident from the experiments when oxygen or carbon dioxide was used. The cotyledons were deep green, almost black, and had an exceptionally good tone. Even after 45 hr. continuous exposure in compressed air no serious injury to the seedlings could be noted. Some of the plants were then transferred to normal conditions for further observation, but no detrimental effects from the unusually high pressure could be detected.

The effect of the radiation on the development of chlorophyll in the cotyledons was evident to the eye. This is confirmed by the figures in Table IV (Fig. 5). The xanthophyll and the carotene also developed well during the 45-hour exposure. This agrees with the findings of Norris*, who irradiated seedlings at normal atmospheric pressure. The amount of carotene present is so slight that it is difficult to compare the results but it is interesting to note that the values obtained are again in harmony with the figures of Norris who claimed a preliminary depression before a regular increment occurred.

Effect of Pressure in the Absence of Radiation

As a further test to determine if excess pressure would seriously injure the plant or inhibit pigment production, plants were placed in the tank and kept for 72 hr. in air at 24 lb. excess pressure. All light was carefully excluded, but the other conditions were the same as in the previous experiments. At the conclusion the plants appeared more vigorous and more turgescent than plants sprouted at the same time and left in the dark chamber.

As was expected, no chlorophyll developed. This again indicates the importance of radiation in the production of chlorophyll. The xanthophyll content was 1.5 mg. per 1000

*Unpublished results.

cotyledons, which was far less than in equally old plants left in the dark chamber at atmospheric pressure. Xanthophyll, therefore, appears to decrease when the pressure is high. The carotene content per 1000 cotyledons was 0.3 mg., which was approximately the same as in equally old plants that served as control.

While compressed air did not kill the plants in the limited time in which they were exposed, it does not seem to be a favorable factor in the production of pigment unless suitable radiation is supplied simultaneously. It seems to increase the absorption of moisture by the plant, but whether the absorbed water has actually entered the cells or merely filled the intercellular spaces is not clear. The pressure favors the condensation of the moisture in the air outside the plant and perhaps within the plant as well.

Third Group

In this series the conditions were the same as in the first except that the period of exposure was extended to 113 hr. It was hoped that any effect produced by a given combination of factors would become more pronounced. The results are given in Table V and are represented graphically in Fig. 6.

It was evident that the plants could not live long in an atmosphere of pure oxygen or carbon dioxide. In both cases (Table V, Fig. 6) the plants were dead and discolored. The mixture of gases in the atmosphere is a favorable medium whether it be at normal pressure or at the excess pressure used in the experiments.

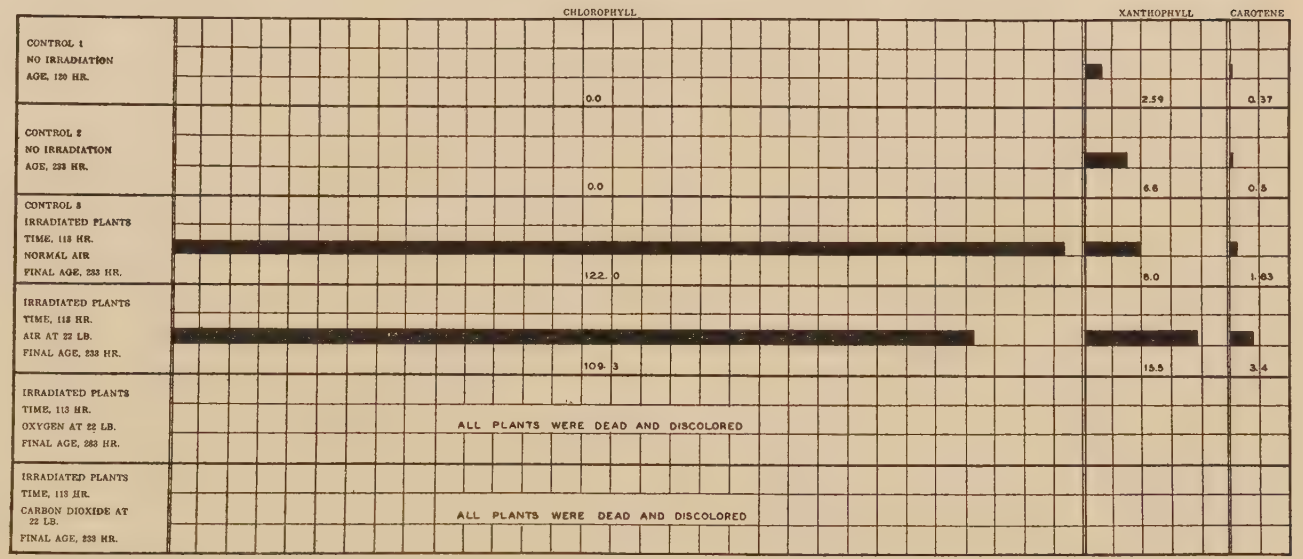
Less chlorophyll developed when the plants were in compressed air than when in air at normal pressure, the difference being about 10 per cent. The reverse was the case for the carotinoid pigments. Considerably more pigment was produced when the excess pressure prevailed. The initial value

TABLE V

The Amount of Pigment Developing in Sunflower Seedlings During 113 hr. Under the Influence of Normal and Compressed Air, Compressed Oxygen and Compressed Carbon Dioxide

Factors	Mg. per 100 g. Wet Weight			Mg. per 10 g. Pulp			Mg. per 1000 Cotyledons			Quotients		
	Chlorophyll	Xanthophyll	Carotene	Chlorophyll	Xanthophyll	Carotene	Chlorophyll	Xanthophyll	Carotene	Ch/X	Ch/Car	X/Car
Control 1 No irradiation Age, 120 hr.	0.0	4.0	0.57	0.0	4.2	0.62	0.0	2.59	0.37	0.0	0.0	7.0
Control 2 No irradiation Age, 233 hr.	0.0	7.8	0.6	0.0	11.7	0.9	0.0	6.6	0.5	0.0	0.0	13.1
Control 3 Irradiated Plants Time, 113 hr. Normal Air Final age, 233 hr.	75.2	4.83	1.15	221.7	14.44	3.32	122.0	8.0	1.83	15.3	66.6	4.5
Irradiated Plants Time, 113 hr. Air at 22 lb. Final age, 233 hr.	60.0	8.6	1.9	143.2	20.4	4.47	109.3	15.5	3.4	7.0	32.0	4.6
Irradiated Plants Time, 113 hr. Oxygen at 22 lb. Final age, 233 hr.	All plants were dead											
Irradiated Plants Time, 113 hr. Carbon Dioxide at 22 lb. Final age, 233 hr.	All plants were dead											

FIG. 6—*The Amount of Pigment Developing in Sunflower Seedlings During 113 hr. Under the Influence of Normal and Compressed Air, Compressed Oxygen and Compressed Carbon Dioxide*



of the xanthophyll may be assumed to have been 2.59 mg. per 1000 cotyledons, and the final value, without irradiation, 6.6 mg. (Table V, Fig. 6, *Controls 1 and 2*). Under normal pressure and irradiation 1.4 mg. more was produced while in compressed air and with radiation the increase over the control was 8.9 mg. Thus the increment under pressure was about 6.3 times as great as the increment under normal conditions.

Similarly, the irradiation produced an increment of 1.33 mg. of carotene per 1000 cotyledons under normal pressure, and but 2.9 more per 1000 cotyledons under excess pressure. This is more than twice the normal increment.

It is evident that the excess pressure did not seriously inhibit the metabolic processes of the plant cells nor prevent the development of the plant pigments. The amount of chlorophyll is almost the same and the production of carotinoid pigment seems to be favored by the excess pressure.

SUMMARY OF RESULTS

In studying the effect of different atmospheres on the development of plant pigments in the cotyledons of sunflowers and Hubbard squash it was found that no chlorophyll developed in the dark during the sprouting period. Xanthophyll and carotene developed without the aid of radiation. Leukophyll or protochlorophyll probably developed in the dark.

The numerical expression of the pigment developed is given best as a function of the number of cotyledons.

The amount of carotinoid pigment developed in the dark increased with the age of the plant.

Experiments with Hubbard squash showed that there is no relation between the development of the chlorophyll and the development of the carotinoid pigments. The carotinoids developed well during radiation when the atmosphere was compressed air and oxygen, but there was a decrement when

the atmosphere was compressed carbon dioxide. The development of the three pigments was roughly proportional to the general metabolism of the plant.

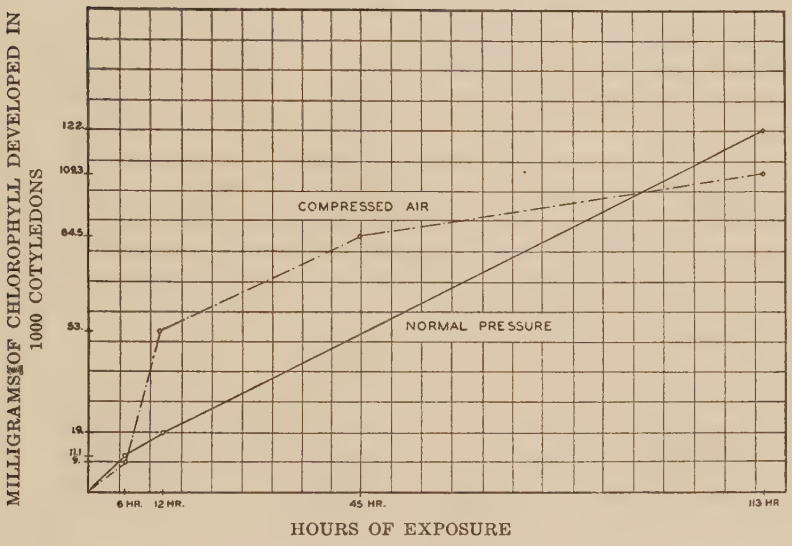
In the preliminary experiments with sunflowers the inhibitory effect of the carbon dioxide was again evident. The xanthophyll developed well in the carbon dioxide atmosphere which was surprising in view of the results with squash. Mere pressure does not inhibit the metabolism nor prevent the development of pigment.

The series of experiments with sunflowers showed that compressed air did not seriously injure the plants during 6 hr. of exposure, but not as much chlorophyll developed as under normal conditions. No chlorophyll developed in the 6 hr. during which the plants were exposed in carbon dioxide. There was no increase of xanthophyll or carotene during the exposure in carbon dioxide. At the end of the short period of exposure there was less present than in equally old plants not exposed. The 45-hour exposure in compressed air stimulated the plants and the amount of chlorophyll developed was considerable. They continued to thrive after the exposure. The amount of xanthophyll also increased under pressure while the development of carotene was but slight. Seventy-two hours of pressure, without light, developed no chlorophyll. The xanthophyll was also less than in equally old plants left in the dark sprouting chamber. The plants under pressure were more turgescent than those not treated, and contained more water than the controls. The compressed air apparently favored the absorption of water and the root development.

The effect of long exposure to the light in compressed atmospheres of various gases showed that plants exposed in oxygen or carbon dioxide died. Equally old plants left in the sprouting chamber remained in normal condition. The plants exposed in compressed air remained vigorous and a considerable amount of chlorophyll developed, but slightly less than control

plants in light and air at normal pressure. More xanthophyll and more carotene developed than in the controls. There appeared to be no relation between the development of the chlorophyll and the carotinoid pigments.

FIG. 7—*The Development of Chlorophyll in Plants Exposed to Light in Compressed Air and in Air at Normal Pressure*

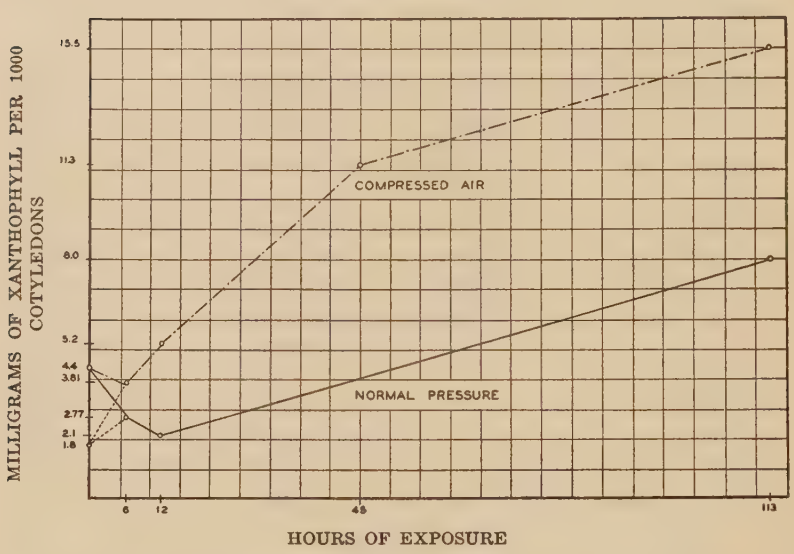


CONCLUSIONS

Compressed air seems to stimulate the plant but the results of the long exposure indicate that fatigue may set in. We may assume that under pressure there is an initial inhibition, a later stimulation, and a final fatigue.

In normal air chlorophyll developed in direct proportion to the time of exposure. Under pressure the production of chlorophyll becomes greater than under normal conditions after 6 hr., but when the time is prolonged it again becomes less.

FIG. 8—*The Development of Xanthophyll in Plants Exposed to Light in Compressed Air and in Air at Normal Pressure*



The compressed air likewise stimulates the production of the carotinoid pigments. The rate decreases with time but the fatigue is not as evident as in the case of chlorophyll.

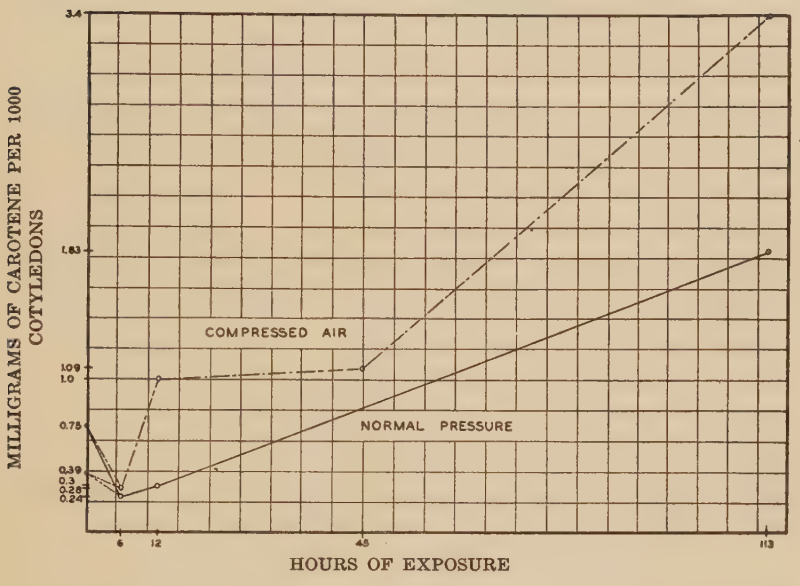
At the conclusion of 72 hr. of treatment in compressed air without light, only 1.5 mg. of xanthophyll developed, which was about the same as the low initial value. As prolonged irradiation had produced an increase of xanthophyll, this last fact is evidence that the light is a factor in the further development of the xanthophyll, even though it is initially present in the absence of light.

The relation between the development of chlorophyll in normal and in compressed air is shown in Fig. 7.

The amount of xanthophyll produced was in proportion to the length of time of exposure to light, and more xanthophyll

was produced in compressed air than in air at normal pressure. There is some indication of fatigue (Fig. 8).

FIG. 9—*The Development of Carotene in Plants Exposed to Light in Compressed Air and in Air at Normal Pressure*



The development of carotene (Fig. 9) was in general similar to the development of the xanthophyll. The amount of pigment formed was proportional to the total quantity of light received. It cannot be stated with certainty which initial control value (Table II, *Control 1*; Table III, *Control 1.*) should be taken as the more nearly ideal, but the general trend is unmistakable whether one or the other is chosen. The apparent or real decrement agrees with the findings of Norris.[5]

As in the case of chlorophyll and xanthophyll, the development of carotene was stimulated by compressed air. The rate

[5]Norris, R. J., *loc. cit.*

of development in compressed air did not seem to be as regular as for the other two pigments, but the quantities were very minute and the probable error correspondingly greater. A straight line drawn from the value given for 6 hr. of exposure to the value for 113 hr., would give a more accurate expression for the increment, so that the 12-hour exposure would appear to be high and the 45-hour to be low.

THE RELATION BETWEEN THE DEVELOPMENT OF CHLOROPHYLL AND TIME OF EXPOSURE TO LIGHT

WILLIAM A. BECK, S.M.,* REV. RICHARD REDMAN AND SISTER M. PETRONELLA SCHROEDER, C.PP.S.

In the highly organized plants chlorophyll is normally found within the living cell.[1] It is actually in the protoplasm and not in the cell sap cavity. This fact suggests a relation between the metabolic activity of the protoplasm and the formation of the plant pigments. In the *Spermatophyta* the chlorophyll is lodged upon specialized bodies known as chloroplasts, which are immersed in the protoplasm. It is generally supposed that the leucoplasts of etiolated plants are identical with the chloroplasts of irradiated plants. Chlorophyll does not actually develop in the chloroplasts until the plant has been exposed to light.[2] Light is a necessary factor for the formation of chlorophyll, and in all probability the formation of chlorophyll is not immediate but is the final product of a chain of precursors which can be formed in the absence of light.

Observation shows that light of medium intensity is best for the development of chlorophyll. Some investigators think that in more intense light the formation and decomposition of chlorophyll occur simultaneously, so that as a net result the greening is not as pronounced as in less intense, diffused light. Ordinary observation shows that plants growing in the shade have more chlorophyll than those growing in direct sunlight. Jönssen[3] verified this colorimetrically by a relatively crude

*Lecturing Professor, University of Dayton, Dayton, Ohio.

[1]Miller, E. C., Plant Physiology, 1931, p. 426.

[2]Palladin, V. I., Plant Physiology, 1923. Eng. transl., B. E. Livingston.

[3]Jönssen, B., *Just's Jahrb.*, 30 II, (1920): 693.

method. Later investigators,[4] with more accurate methods, confirmed his findings. Using the improved methods of Willstaetter[5] (1914) for the separation of plant pigments, Stafelt and Luhdegardh verified the work of Lubimenko that "light" plants have less chlorophyll than "shade" plants.

Temperature is another factor affecting the rate of the production of chlorophyll. In etiolated plants greening occurs most quickly between 18° C. and 30° C. According to Wiessner[6] chlorophyll is produced twice as fast within that range of temperature as at 10° C. The presence of oxygen is also essential for the production of chlorophyll. Etiolated leaves in an atmosphere devoid of oxygen remain yellow even when exposed to light. Besides the above mentioned factors, there are others, such as mineral nutrients and carbohydrates, which are assumed to be necessary for the formation of the precursors of chlorophyll. Their immediate transformation into chlorophyll, however, is considered to be dependent on light.

In a series of experiments to study the effect of some unusual factors upon the development of plant pigments, the authors observed a relationship between the total amount of chlorophyll developed in the cotyledons and the quantity of light received.

The object of the present series of experiments was to verify a relation between the amount of chlorophyll developed and the time of exposure to a constant light source.

EXPERIMENTAL

Sunflower seeds were sprouted in small pots filled with soil to which a little Knopp's solution had been added. The pots

[4]Lubimenko, V. N., *Rev. Gen. Botan.*, **17** (1905): 381; **20** (1908): 162, 217, 253, 285.

[5]Willstaetter, R., and Stoll, A., Untersuchungen ueber Chlorophyll, Berlin, 1913.

[6]Wiessner, J., *Sitzber. Akad. Wiss. Wien*, **69** (1877): 327.

were sunk in German peat contained in a shallow pan. Over this pan a hood was placed which was light-proof but permitted the circulation of air. This sprouting chamber was further placed in a light-proof compartment in which were maintained a temperature of 26° to 28° C. and a relative humidity of 95 to 100 per cent.

When the plants were 90 hr. old they were placed in a pressure chamber, previously employed and described.[7] Atmospheric pressure was maintained during the exposures. The

TABLE I

Chlorophyll Yield as a Function of Time of Exposure

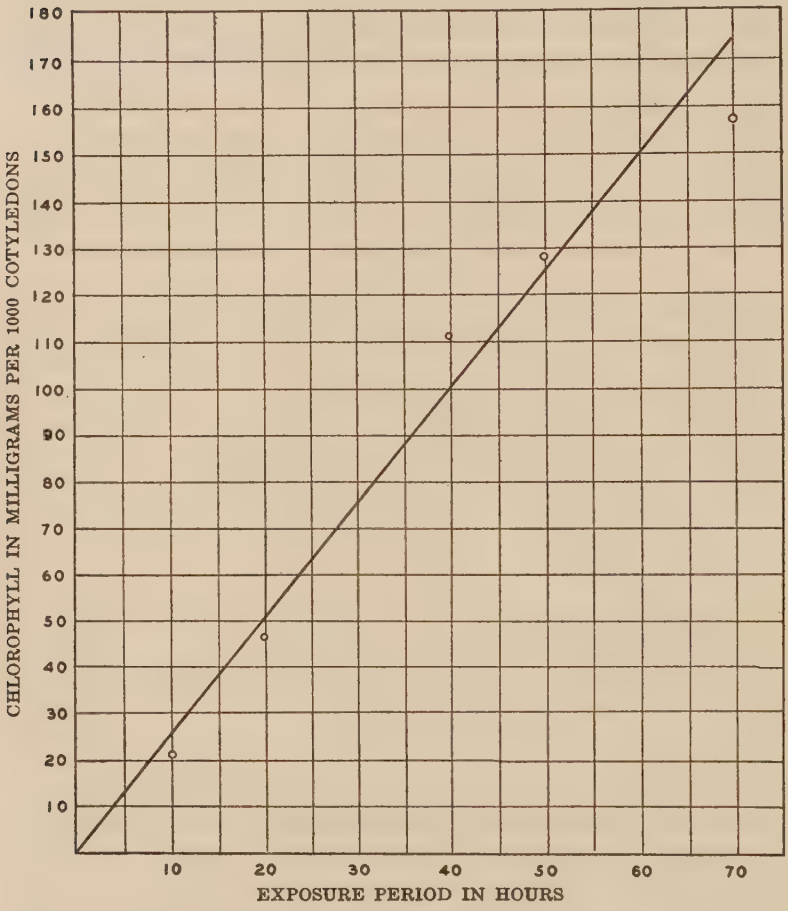
Time of Exposure to Light	Actual Yield of Test Plants	Ideal Yield of Plants	Deviation	Percentage Deviation
10 hr.	21.375 mg.	23.93 mg.	−2.55 mg.	−10.6
20 "	47.14 "	47.86 "	−0.72 "	−1.5
40 "	101.27 "	95.72 "	+5.55 "	+5.7
50 "	127.82 "	119.65 "	+8.17 "	+6.7
70 "	157.20 "	167.51 "	−10.31 "	−6.1

time of exposure in the different experiments was 10, 20, 40, 50, or 70 hr. The light was admitted to the plants through a thick, shatter-proof glass, care being taken to place the lamp always at the same distance above the plants. Between the plants and the lamp a cooling cell was inserted through which water passed continuously, thus preventing the plants from becoming overheated. The temperature in the chamber was kept between 25° and 27° C.

After each exposure the cotyledons were clipped and the chlorophyll was extracted by a modification of the Willstaetter-

[7] Beck, W. A., *this issue*, p. 217.

FIG. 1—*Graph of the Rate of Development of Chlorophyll in Sunflower Seedlings During Exposure to Light*



Schertz[8] method and measured by a photodensitometer.[9] Control plants of the same age as the experimental plants were left in the dark. Pigment extraction was carried out for these also but there was no yield of chlorophyll.

[8]Schertz, F. M., *Plant Physiol.*, 3 (1928): 211.

[9]Beck, W. A., *Science*, 85 No. 2206 (1937): 368.

The chlorophyll yields expressed in milligrams per 1000 cotyledons are assembled in Table I.

Since chlorophyll did not develop in any of the control plants they are not included in the table.

The total number of hours of exposure for all the plants listed in Table I was 190 hr. The total chlorophyll yield was 454.805 mg. Accordingly, the average rate of development was 2.393 mg. per hour. The ideal yield calculated on the basis of this average rate and the deviation of the actual amount from the ideal amount are given in columns 3, 4 and 5. In Fig. 1 the chlorophyll developed is plotted against the time of exposure.

DISCUSSION

The chlorophyll yield for the 10-hour, 20-hour, 40-hour, and 50-hour exposures varies slightly from the ideal yield for the same periods (Table I, Fig. 1). This is, however, within the limits of probable experimental error. The 70-hour exposure, however, shows a deviation of -10.31 from the ideal yield. This is beyond the limit of probable experimental error and is no doubt due to some factor that has not been taken into consideration. Further experiments, in which seedlings were exposed for 80 hr. and 100 hr., gave a chlorophyll yield per hour which was consistently lower than the mean value given above (2.393 mg. per hr. per 1000 cotyledons).

It is surprising that the decline occurred before the 100-hour period had been reached, since the results of prior experiments[10] indicated only a slight decline. An exhaustive study of the effect was not made nor were many experiments performed. Then, too, the number of hours of exposure in these experiments was widely different.

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[10]Beck, W. A., *loc. cit.*

Analysis of various factors which could possibly have been responsible for this decline of chlorophyll production culminated in the suspicion that the small size of the seeds used in the experiments in which the exposure was above 70 hr. was responsible for the relatively small quantity of pigment produced. In a given lot of seeds the smaller seeds invariably tend to collect below while the larger ones remain at the top, so that, other things being equal, the larger seeds will be selected for planting before the smaller ones even though a great number of approximately equally-sized seeds are chosen from the beginning.

A series of experiments was carried out to determine the relation between the size and weight of seeds and the amount of chlorophyll developed in the cotyledons of their seedlings, the results of which are published separately. The results led to the conclusion that smaller seeds produce seedlings that yield, under like conditions, less chlorophyll when irradiated for a given time, than do seedlings produced by larger seeds. Thus the negative deviation of the actual yield from that of the ideal yield for the 70-hour exposure seems to be satisfactorily explained. The possibility still remains that a direct relationship does not exist between the amount of chlorophyll developed and the time of exposure to light.

Within the limits of the experiments it may be concluded that the amount of chlorophyll developed in the cotyledons is proportional to the quantity of light received. From these results it may not, however, be concluded that this rate of development per hour will be maintained for an unlimited length of time, since the capacity of the cotyledons, so far as bulk and mass are concerned, is limited, and consequently a maximum of chlorophyll production should be reached at some time. Probably this would not occur in our chamber before 100 hr. of exposure.

CONCLUSION

Within the limits of the experiments, the chlorophyll produced in the cotyledons was proportional to the quantity of light received.

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